INFLUENCE OF SILDENAFIL AND DONEPEZIL ADMINISTRATION ON THE SERUM REDOX BALANCE IN EXPERIMENTALLY INDUCED LOWER LIMB CRITICAL ISCHEMIA

MIHAELA IOANA CONSTANTINESCU[1], DAN PETRU CONSTANTINESCU[2], AUREL ANDERCOU[1], ION AUREL MIRONIUC[1]

[1] 2nd Surgical Clinic, County Clinic Hospital, Cluj-Napoca, Romania
[2] 4th Surgical Clinic, County Clinic Hospital, Cluj-Napoca, Romania

Abstract

Introduction. *Chronic lower limb ischemia (CLLI) leads to endothelial cell dysfunctions and endothelial lesions. The use of substances that release nitric oxide and activate endothelial nitric oxide synthase has proved to be useful in increasing angiogenesis and arteriogenesis under critical ischemia conditions.*

Objectives. *To investigate the therapeutic effect of Sildenafil and Donepezil with a vasodilating action in experimentally induced CLLI and on serum redox homeostasis.*

Material and method. *The research was performed in 3 groups of rats (n=10 animals/group) with experimentally induced CLLI: group I – control group; group II – animals treated postoperatively with a therapeutic dose of sildenafil, and group III – animals treated postoperatively with a therapeutic dose of donepezil. Oxidative stress (OS) indicators (malondialdehyde - MDA, protein carbonyls - PC), antioxidant (AO) defense indicators (reduced glutathione - GSH and oxidized glutathione - GSSH), and ceruloplasmin (CP) were determined on days 7, 14, 21 and 30. Statistical processing was performed using the Excel application (Microsoft Office 2007), with the StatsDirect v.2.7.2 software.*

Results. *Changes in OS were evidenced in all groups on account of a decrease in MDA and PC. The greatest OS decrease in all groups was on day 30. AO defence changes were represented by decreased levels of GSH and GSSG in all groups, at the studied moments. Intracellular AO defense in the cytosol, nucleus and mitochondria was similar in all groups, (decreased GSH, GSSG and GSH/GSSG ratio). We found increased extracellular levels of GSH, GSSG, and CP and increased extracellular GSH/GSSG ratio at level compared to values on day 7.*

Conclusions. *1) The administration of sildenafil (group II) and donepezil (group III) has favorable effects on reducing OS in experimentally induced CLLI. 2) Sildenafil and Donepezil administration stimulates extracellular AO defense on account of CP. 3) Sildenafil and Donepezil administration influences intracellular redox homeostasis on account of the GSH/GSSG couple, the major redox buffer in the body.*

Keywords: chronic ischemia, lower limb, Sildenafil, Donepezil, oxidative stress, antioxidant defence.

Manuscript received: 01.08.2013
Received in revised form: 10.09.2013
Accepted: 11.09.2013
Address for correspondence: ioanaconstantinescu2003@yahoo.com

Introduction

Many experimental studies have shown that both ischemia and ischemia-reperfusion syndrome are associated with oxidative stress (OS) through the production of reactive oxygen species (ROS) [1,2]. Repeated ischemia and reperfusion episodes may trigger lesions at the level of endothelial cells, mitochondria, muscle fibers, and neuronal axons. These oxidative lesions will initiate chronic changes in the metabolism and structure of muscle fibers, resulting in the loss of their function, which can be entirely attributed to the reduction of blood flow and oxidative lesions [3].

Endothelial cell dysfunction is an early event which will induce lesions in the vascular wall. This will result in the impairment of the capacity of endothelial cells to maintain vascular function and homeostasis [4].

Nitric oxide (NO) is known as a physiologically vasoactive agent, involved in the proliferation and survival of endothelial cells, the increase of their mobility and the activation of mechanisms necessary for angiogenesis [5,6,7]. It plays a role in the regulation of blood pressure, the mediation of vascular permeability, reactive vasodilation, blood flow-dependent dilation and endothelium-dependent relaxing responses to acetylcholine (Ach). It has antiaggregant properties, stimulates endogenous fibrinolysis and maintains the antiinflammatory state of the vascular wall [8].

The use of substances that release NO and activate endothelial nitric oxide synthase (eNOS), such as statins and vascular endothelial growth factor A (VEGF-A), has proved to be useful in increasing angiogenesis and arteriogenesis under critical ischemia conditions [9,10]. However, substances that release nitric oxide may also have negative effects. NO excess associated with the uncoupling of eNOS results in the formation of superoxide and peroxynitrite radicals, which leads to the extension of the surface of the affected tissues in the ischemia-reperfusion syndrome [11].

NO activates the guanylate cyclase enzyme and increases the concentration of cyclic guanosine monophosphate (cGMP), inducing smooth muscle relaxation. Sildenafil is a selective enzyme inhibitor, which enhances the effect of NO through the inhibition of type 5 phosphodiesterase (PDE5), which is responsible for the degradation of cGMP and is mainly found in the smooth muscle of the cavernous body, but also in smooth vascular, visceral and skeletal muscles. Sildenafil treatment may have a protective action in pathological tissue ischemia, through the increase in the tissue level of cGMP and the stimulation of ischemia-induced angiogenesis, without the implication of eNOS [12].

Donepezil, a specific and reversible acetylcholinesterase inhibitor, induces an increase in Ach levels. Ach causes a reduction of blood pressure and vasodilation through an indirect mechanism: it acts on endothelial cells that release the endothelial relaxing factor NO, through cGMP. Ach is also an activating modulating factor for the increase of Ca^{2+} concentration, with stimulating effects on eNOS and NO synthesis. On the other hand, a study on the effects of Ach on myocardial cells has shown that Ach prevents hypoxia-induced cell death [13]. However, there are also data demonstrating the negative effects of systemic Ach administration, due to the induction of bronchospasm and mucus hypersecretion at the level of the airways. In cardiovascular patients, Donepezil acts as a therapeutic agent that activates angiogenesis [14]. This is why we used it for testing its effect on rats with experimentally induced chronic lower limb ischemia (CLLI).

Objectives

The objectives of this study were to investigate the therapeutic effect of Sildenafil and Donepezil in experimentally induced CLLI and their influence on serum redox homeostasis.

Material and method

The experimental study was an interventional, prospective, analytical pilot study, with a duration of 30 days. The research was performed in the Experimental Laboratory of the Department of Physiology of the "Iuliu Haţieganu" University of Medicine and Pharmacy Cluj-Napoca, on white Wistar rats with a weight of 280-300 g, maintained under adequate vivarium conditions; with the approval of the Ethics Board No. 681/18.12.2012. During the experimental research, the legislation in force regarding animal protection was respected; at the end of the experiment, the animals were euthanized.

a) Groups

The groups (n=10 animals/group) were the following:

- group I – control – operated, untreated animals;
- group II – operated animals, treated postoperatively with a therapeutic dose of Sildenafil, 10 mg/kg body weight/day, by oropharyngeal gavage, for 30 days (day 5-day 34); we utilised Sildenafil powder resulting from 100 mg pills dissolved in drinking water, to a final concentration of 40 µg/ml. A resulting dose of 10 mg/kg/day of Sildenafil was achieved, to reach a therapeutic dosing level that would be comparable in man because of the short half life in rats (1 hour in rats versus 4 hour in man) [15];
- group III – operated animals, treated postoperatively with a therapeutic dose of Donepezil, 5 mg/kg/day, by oropharyngeal gavage, for 30 days (day 5-day 34); we utilised Donepezil powder resulting from 10 mg pills dissolved in drinking water, to a final concentration of 50 µg/ml. A resulting dose of 5 mg/kg/day of Donepezil was achieved, to reach a therapeutic dosing level that would be comparable in man. This dose was initially determined to clearly show the expected effects without producing adverse effects in rats [14].

We used Sildenafil (Viagra®, Pfizer 50 mg/compr) and Donepezil (Aricept®, Pfizer 10 mg/tabl).

b) Methods

- experimental surgical

The animals were operated in the Experimental Laboratory of the Department of Physiology of the "Iuliu Haţieganu" University of Medicine and Pharmacy, using a procedure adapted from Colleran et al. and Yang et al. [16,17]. CCLI was induced by the ligation of the common femoral artery, proximally to the origin of the deep femoral artery (day 0), followed by the ligation of the common iliac artery (day 4). The rats were anesthetized with ketamine 100 mg/kg and xylazine 8 mg/kg.

The control of ischemia was performed on day 5 through:

- clinical score (0=normal mobility, 1=pallor, limping, 2=gangrenous tissue limited to less than half of the leg, without lower limb necrosis, 3=gangrenous tissue limited to less than half of the leg, with lower limb necrosis, 4=gangrenous tissue extended to more than half of the leg, 5=extensive necrosis of the lower limb [5], and
- imaging, by Doppler ultrasound.

- biochemical

Serum determinations from venous blood collected from the retro-orbital vein were performed in the Laboratory for the Study of Oxidative Stress of the Department of Physiology of the "Iuliu Haţieganu" University of Medicine and Pharmacy Cluj-Napoca. The selected moments were postoperative days 7, 14, 21, 30.

The following data were measured:

✓ Oxidative stress indicators

- *malondialdehyde* (MDA) (using the fluorescence method, according to Conti). Concentration values were expressed in nmol/ml, for serum [17].
- *protein carbonyls* (PC) (using the method of Reznick and Packer). The results were expressed in µmol/ml, for serum [19].

✓ Antioxidant defense indicators

- *reduced glutathione* (GSH) and *oxidized glutathione* (GSSG) (using the method of Hu); values were expressed in µmol/l or nmol/ml in the serum [20].

✓ Ceruloplasmin (CP) (using Ravin's method); values were expressed in mg/100 ml [21].

c) Statistical analysis

Descriptive statistical elements were calculated, and data were presented using centrality, location and distribution indicators.

For testing normal distribution, the Shapiro-Wilk W test was used. For statistical data analysis, in the case of normal distribution data, the (Student) t test was used, variances being tested with the Levene test. In the case of non-uniform distribution values, the non-parametric Mann-Whitney (U) test for two unpaired samples and the Wilcoxon test for two paired samples were used.

The significance threshold for the tests used was $\alpha=0.05$ (5%): $0.01<p<0.05$ – statistically significant difference; $0.001<p<0.01$ – very statistically significant difference; $p<0.001$ – highly statistically significant difference; $p>0.05$ – statistically insignificant difference.

Statistical processing was carried out with the Excel application (Microsoft Office 2007) and with the StatsDirect v.2.7.2 software.

Results

Comparative statistical analysis by moments between the groups

a. Malondialdehyde

The statistical analysis of *MDA* values (Table I), *considering all studied moments,* evidenced highly statistically significant differences between at least two of the studied moments in groups I ($p=4.91x10^{-7}$), II ($p=0.0003$) and III ($p=7.54x10^{-6}$).

The statistical analysis of *MDA* values *for paired samples* showed:

- in group I – highly statistically significant differences between days 7-21 and 14-30 ($p<0.001$) and very statistically significant differences between days 7-30 and 14-21 ($p<0.01$);

Table I. Comparative analysis of MDA values (measured in nmol/ml) at the studied moments and statistical significance.

Group	Moment	Mean	SE	Median	SD	Min.	Max.	*Statistical significance (p) between moments*	
I	**Day 7**	2.0713	0.1252	1.955	0.3540	1.71	2.82	D_7-D_{14}: 0.5618	D_{14}-D_{30}: **0.0002**
	Day 14	1.9688	0.0738	1.985	0.2088	1.58	2.27	D_7-D_{21}: **0.0002**	D_{21}-D_{30}: 0.6342
	Day 21	1.105	0.1178	1.15	0.3333	0.57	1.56	D_7-D_{30}: **0.0028**	
	Day 30	1	0.1720	0.9	0.4866	0.4	1.87	D_{14}-D_{21}: **0.0016**	
II	**Day 7**	1.9475	0.0808	1.925	0.2286	1.59	2.35	D_7-D_{14}: 0.711	D_{14}-D_{30}: **0.0078**
	Day 14	1.9875	0.0569	1.92	0.1609	1.8	2.22	D_7-D_{21}: **0.0127**	D_{21}-D_{30}: 0.4609
	Day 21	1.195	0.1969	1.21	0.5569	0.32	2.1	D_7-D_{30}: **0.0078**	
	Day 30	1.0213	0.1493	0.865	0.4224	0.58	1.6	D_{14}-D_{21}: **0.0034**	
III	**Day 7**	1.6375	0.0745	1.675	0.2108	1.25	1.95	D_7-D_{14}: **0.002**	D_{14}-D_{30}: **0.0001**
	Day 14	2.3788	0.1648	2.23	0.4662	1.65	3.04	D_7-D_{21}: 0.2933	D_{21}-D_{30}: **0.0024**
	Day 21	1.8025	0.1345	1.7	0.3803	1.33	2.54	D_7-D_{30}: **0.0278**	
	Day 30	1.1588	0.1410	1.26	0.3987	0.53	1.62	D_{14}-D_{21}: **0.0025**	
Statistical significance (p) between groups		D_7 (I-II): 0.4224		D_{14} (I-II): 0.8436		D_{21} (I-II): 0.7024		D_{30} (I-II): 0.9804	
		D_7 (I-III): **0.0126**		D_{14} (I-III): **0.0465**		D_{21} (I-III): **0.0016**		D_{30} (I-III): 0.488	
		D_7 (II-III): **0.0136**		D_{14} (II-III): 0.0515		D_{21} (II-III): **0.0256**		D_{30} (II-III): 0.6454	

• in group II – statistically significant differences between days 7-21 ($p<0.05$) and very statistically significant differences between days 7-30, 14-21 and 14-30 ($p<0.01$);

• in group III – highly statistically significant differences between days 14-30 ($p<0.001$), very statistically significant differences between days 7-14, 14-21 and 21-30 ($p<0.01$), and statistically significant differences between days 7-30 ($p<0.05$).

The statistical analysis of *MDA* values, *considering all groups at a certain moment*, evidenced statistically significant differences between at least two of the groups on day 7 ($p=0.0128$) and on day 14 ($p=0.0239$), and very statistically significant differences between at least two of the groups on day 21 ($p=0.0081$). On day 30, no statistically significant differences between any of the groups were found ($p=0.7241$).

The statistical analysis of *MDA* values *for unpaired samples* showed:

• on day 7 – statistically significant differences between groups I-III and II-III ($p<0.05$);

• on day 14 – statistically significant differences between groups I-III ($p<0.05$);

• on day 21 – very statistically significant differences between groups I-III ($p<0.01$) and statistically significant differences between groups II-III ($p<0.05$). On day 30, there were no statistically significant differences between the studied groups.

b.. Protein carbonyls

The statistical analysis of *PC* values (Table II), *considering all studied moments*, indicated statistically significant differences between at least two of the studied moments in group I ($p=0.0114$). In groups II and III, there were no statistically significant differences between any of the studied moments ($p=0.3348$ and $p=0.0533$, respectively). Although in some cases, considering all studied moments, no statistically significant differences between these could be found, the statistical analysis of *PC* values *for paired samples* evidenced:

• in group I – statistically significant differences between days 7-21 and 14-21 ($p<0.05$) and very statistically significant differences between days 21-30 ($p<0.01$);

• in group III – very statistically significant differences between days 14-21 ($p<0.01$). In group II, no statistically significant differences between the studied days were found.

The statistical analysis of *PC* values, *considering*

Table II. Comparative analysis of PC values (measured in nmol/ml) at the studied moments and statistical significance.

Group	Moment	Mean	SE	Median	SD	Min.	Max.	*Statistical significance (p) between moments*	
I	**Day 7**	1.0125	0.1329	0.9200	0.3758	0.65	1.8	D_7-D_{14}: 0.2251	D_{14}-D_{30}: 0.1147
	Day 14	0.8263	0.0439	0.825	0.1242	0.69	1	D_7-D_{21}: **0.0357**	D_{21}-D_{30}: **0.0093**
	Day 21	0.5588	0.0683	0.51	0.1933	0.34	0.89	D_7-D_{30}: 0.9847	
	Day 30	1.0088	0.1303	1.055	0.3685	0.44	1.5	D_{14}-D_{21}: **0.0106**	
II	**Day 7**	0.7488	0.0995	0.72	0.2815	0.43	1.24	D_7-D_{14}: 0.0781	D_{14}-D_{30}: 0.4375
	Day 14	1.0275	0.0806	0.935	0.2279	0.81	1.5	D_7-D_{21}: 0.9375	D_{21}-D_{30}: 0.25
	Day 21	0.7425	0.1135	0.73	0.3210	0.43	1.21	D_7-D_{30}: 0.267	
	Day 30	0.9625	0.1449	0.865	0.4099	0.52	1.65	D_{14}-D_{21}: 0.3125	
III	**Day 7**	0.9975	0.1425	0.9	0.4029	0.49	1.86	D_7-D_{14}: 0.3041	D_{14}-D_{30}: 0.2421
	Day 14	0.8025	0.0651	0.785	0.1841	0.61	1.1	D_7-D_{21}: 0.0845	D_{21}-D_{30}: 0.1068
	Day 21	1.275	0.0925	1.21	0.2616	0.98	1.7	D_7-D_{30}: 0.9691	
	Day 30	0.9888	0.1395	0.835	0.3947	0.56	1.67	D_{14}-D_{21}: **0.0068**	
Statistical significance (p) between groups		D_7 (I-II): 0.1362		D_{14} (I-II): 0.0605		D_{21} (I-II): 0.2328		D_{30} (I-II): 0.8158	
		D_7 (I-III): 0.9397		D_{14} (I-III): 0.7674		D_{21} (I-III): **3.07 x 10^{-05}**		D_{30} (I-III): 0.918	
		D_7 (II-III): 0.1759		D_{14} (II-III): 0.065		D_{21} (II-III): **0.003**		D_{30} (II-III): 0.898	

Table III. Comparative analysis of GSH values (measured in nmol/ml) at the studied moments and statistical significance.

Group	Moment	Mean	SE	Median	SD	Min.	Max.	*Statistical significance (p) between moments*	
I	**Day 7**	7.6675	0.3437	7.67	0.9720	6	9.15	D_7-D_{14}: 0.1953	D_{14}-D_{30}: 0.6406
	Day 14	6.6263	0.5171	6.355	1.4625	4.99	10	D_7-D_{21}: **0.0078**	D_{21}-D_{30}: 0.0156
	Day 21	3.0688	0.5184	2.65	1.4663	1.6	6.4	D_7-D_{30}: **0.0116**	
	Day 30	6.0931	0.2689	6.2	0.7607	5	7.3	D_{14}-D_{21}: **0.0156**	
II	**Day 7**	7.4175	0.6085	7.4	1.7212	5	11	D_7-D_{14}: 0.3092	D_{14}-D_{30}: 0.2457
	Day 14	5.9625	1.0438	5.275	2.9524	2.19	11.35	D_7-D_{21}: **0.0095**	D_{21}-D_{30}: 0.4865
	Day 21	3.825	0.7088	3.45	2.0048	1.72	7.4	D_7-D_{30}: **0.0019**	
	Day 30	4.5063	0.4091	4.7	1.1571	2.65	5.65	D_{14}-D_{21}: 0.1850	
III	**Day 7**	6.4725	0.2585	6.47	0.7310	5.43	7.58	D_7-D_{14}: **1.4 x 10^{-05}**	D_{14}-D_{30}: 0.2608
	Day 14	3.4813	0.3210	3.325	0.9080	2.49	5.27	D_7-D_{21}: **0.00098**	D_{21}-D_{30}: 0.8945
	Day 21	4.075	0.3622	3.95	1.0243	2.75	5.95	D_7-D_{30}: **0.02**	
	Day 30	4.1563	0.7237	3.675	2.0469	2.05	8	D_{14}-D_{21}: 0.1828	
Statistical significance (p) between groups		D_7 (I-II): 0.7273		D_{14} (I-II): 0.1949		D_{21} (I-II): 0.5054		D_{30} (I-II): **0.0071**	
		D_7 (I-III): **0.0156**		D_{14} (I-III): **0.0003**		D_{21} (I-III): **0.0406**		D_{30} (I-III): **0.0334**	
		D_7 (II-III): 0.1867		D_{14} (II-III): 0.0527		D_{21} (II-III): 0.7599		D_{30} (II-III): 0.6818	

all groups at a certain moment, showed highly statistically significant differences between at least two of the groups on day 21 (p=0.0009). On days 7, 14 and 30, there were no statistically significant differences between any of the groups (p=0.2746, p=0.0833, and p=0.9723, respectively).

The statistical analysis of *PC* values *for unpaired samples* evidenced:

- on day 21 – highly statistically significant differences between groups I-III (p<0.001) and very statistically significant differences between groups II-III (p<0.01). On days 7, 14 and 30, no statistically significant differences between the studied groups were found.

c. Reduced glutathione

The statistical analysis of *GSH* values (Table III), *considering all studied moments,* revealed highly statistically significant differences between at least two of the studied moments in groups I and III (p=0.0004 and p=0.0004, respectively) and very statistically significant differences between at least two of the studied moments in group II (p=0.0081).

The statistical analysis of *GSH* values *for paired samples* evidenced:

- in group I – very statistically significant differences between days 7-21 (p<0.01) and statistically significant differences between days 7-30, 14-21 and 21-30 (p<0.05);
- in group II – very statistically significant differences between days 7-21 and 7-30 (p<0.01);
- in group III – highly statistically significant differences between days 7-14, 7-21 (p<0.001) and statistically significant differences between days 7-30 (p<0.05).

The statistical analysis of *GSH* values**,** *considering all groups at a certain moment,* revealed very statistically significant differences between at least two of the groups on day 14 (p=0.0032) and statistically significant differences between at least two of the groups on day 30 (p=0.0295). On days 7 and 21, no statistically significant differences between any of the groups were found (p=0.1418 and p=0.1612, respectively).

Although in some cases, considering all studied groups, there were no statistically significant differences between these, the statistical analysis of *GSH* values *for unpaired samples* evidenced:

- on day 7 – statistically significant differences between groups I-III (p<0.05);
- on day 14 – highly statistically significant differences between groups I-III (p<0.001);
- on day 21 – statistically significant differences between groups I-III (p<0.05);
- on day 30 – statistically significant differences between groups I-III (p<0.05) and very statistically significant differences between groups I-II (p<0.01).

d. Oxidized glutathione

The statistical analysis of *GSSG* values (Table IV), *considering all studied moments,* showed highly statistically significant differences between at least two of the studied moments in group I (p=0.0003). No statistically significant differences were found between the studied moments in groups II and III.

Although in some cases, considering all studied moments, there were no statistically significant differences between these, the statistical analysis of *GSSG* values *for paired samples* indicated:

- in group I – statistically significant differences between days 7-14 and 7-21 (p<0.05) and very statistically significant differences between days 7-30 and 14-30 (p<0.01);
- in group II – statistically significant differences between days 7-30 (p<0.05). In group III, there were no statistically significant differences between the studied days.

The statistical analysis of *GSSG* values**,** *considering all groups at a certain moment,* showed very statistically significant differences between at least two of the groups on day 7 (p=0.0026). On days 14, 21 and 30, there were no statistically significant differences between any of the groups (p=0.2252, p=0.3949, and p=0.2283, respectively).

Table IV. Comparative analysis of GSSG values (nmol/ml) at the studied moments and statistical significance.

Group	Moment	Mean	SE	Median	SD	Min.	Max.	*Statistical significance (p) between moments*	
I	**Day 7**	1.67	0.0609	1.69	0.1724	1.4	1.88	D_7-D_{14}: **0.0107**	D_{14}-D_{30}: **0.0078**
	Day 14	1.3988	0.0551	1.415	0.1558	1.21	1.61	D_7-D_{21}: **0.0219**	D_{21}-D_{30}: 0.0781
	Day 21	1.325	0.1306	1.3	0.3694	0.6	1.8	D_7-D_{30}: **0.0078**	
	Day 30	0.875	0.0526	0.8	0.1488	0.8	1.2	D_{14}-D_{21}: 0.6721	
II	**Day 7**	1.3325	0.0534	1.32	0.1511	1.12	1.6	D_7-D_{14}: 0.0781	D_{14}-D_{30}: 0.2969
	Day 14	1.2188	0.0837	1.16	0.2367	1	1.74	D_7-D_{21}: 0.25	D_{21}-D_{30}: 0.5469
	Day 21	1.165	0.1309	1.16	0.3701	0.72	1.6	D_7-D_{30}: **0.0228**	
	Day 30	1.05	0.0732	1	0.2070	0.8	1.4	D_{14}-D_{21}: 0.7422	
III	**Day 7**	1.3125	0.0496	1.27	0.1402	1.18	1.5	D_7-D_{14}: 0.7422	D_{14}-D_{30}: 0.3828
	Day 14	1.2225	0.1425	1.245	0.4031	0.5	1.74	D_7-D_{21}: 0.1563	D_{21}-D_{30}: 0.2969
	Day 21	1.14	0.07329	1.19	0.2073	0.7	1.36	D_7-D_{30}: 0.0781	
	Day 30	1	0.1363	0.8	0.3854	0.6	1.6	D_{14}-D_{21}: 0.691	
Statistical significance (p) between groups		D_7 (I-II): **0.00095**		D_{14} (I-II): **0.0468**		D_{21} (I-II): 0.4884		D_{30} (I-II): 0.0884	
		D_7 (I-III): **0.0011**		D_{14} (I-III): 0.2784		D_{21} (I-III): 0.2424		D_{30} (I-III): 0.7716	
		D_7 (II-III): 0.7792		D_{14} (II-III): 0.9043		D_{21} (II-III): 0.7052		D_{30} (II-III): 0.3678	

Although in some cases, considering all studied groups, no statistically significant differences between these could be found, the statistical analysis of *GSSG* values *for unpaired samples* evidenced:

• on day 7 – highly statistically significant differences between groups I-II ($p<0.001$) and very statistically significant differences between groups I-III ($p<0.01$);

• on day 14 – statistically significant differences between groups I-II ($p<0.05$). On days 21 and 30, there were no statistically significant differences between the studied groups.

e. Reduced glutathione/oxidized glutathione ratio

The statistical analysis of *GSH/GSSG* values (Table V), *considering all studied moments,* indicated highly statistically significant differences between at least two of the studied moments in group I ($p<0.0001$). No statistically significant differences were found between any of the studied moments in groups II and III ($p=0.4996$ and $p=0.0541$, respectively).

Although in some cases, considering all studied moments, no statistically significant differences between these could be evidenced, the statistical analysis of *GSH/GSSG* values *for paired samples* showed:

• in group I – highly statistically significant differences between days 14-30 and 21-30 ($p<0.001$), very statistically significant differences between days 7-30 and 14-21 ($p<0.01$) and statistically significant differences between days 7-21 ($p<0.05$);

• in group III – highly statistically significant differences between days 7-14 ($p<0.001$). In group II, there were no statistically significant differences between the studied days.

The statistical analysis of *GSH/GSSG* values*, considering all groups at a certain moment,* showed statistically significant differences between at least two of the groups on day 7 ($p=0.0486$) and on day 30 ($p=0.0353$). On days 7, 14 and 21, no statistically significant differences between any of the groups were found ($p=0.0962$ and $p=0.2128$, respectively).

The statistical analysis of *GSH/GSSG* values *for unpaired samples* indicated:

• on day 30 – very statistically significant differences between groups I-II ($p<0.01$). On days 14 and 21, no statistically significant differences between the studied groups were found.

Table V. Comparative analysis of GSH/GSSG values at the studied moments and statistical significance.

Group	Moment	Mean	SE	Median	SD	Min.	Max.	*Statistical significance (p) between moments*	
I	**Day 7**	4.6463	0.3018	4.555	0.8536	3.75	6.53	D_7-D_{14}: 0.1563	D_{14}-D_{30}: **0.0007**
	Day 14	3.9913	0.1951	3.935	0.5519	3.19	5.15	D_7-D_{21}: **0.0156**	D_{21}-D_{30}: 0.**0001**
	Day 21	2.2575	0.3313	1.99	0.9371	1.29	3.75	D_7-D_{30}: **0.0078**	
	Day 30	7.1288	0.5163	7.37	1.4603	5	9.12	D_{14}-D_{21}: **0.0033**	
II	**Day 7**	5.5475	0.3502	5.63	0.9905	3.57	6.87	D_7-D_{14}: 0.681	D_{14}-D_{30}: 0.6
	Day 14	5.0988	0.9630	4.785	2.7236	2.02	9.46	D_7-D_{21}: 0.1902	D_{21}-D_{30}: 0.7412
	Day 21	3.9675	1.0231	3.14	2.8936	1.08	9.02	D_7-D_{30}: 0.0894	
	Day 30	4.4663	0.5401	4.3	1.5275	2.12	6.93	D_{14}-D_{21}: 0.4906	
III	**Day 7**	4.9363	0.1478	4.965	0.4180	4.38	5.67	D_7-D_{14}: **0.0003**	D_{14}-D_{30}: 0.2045
	Day 14	3.1525	0.3532	3.11	0.9989	1.55	4.98	D_7-D_{21}: 0.25	D_{21}-D_{30}: 0.4609
	Day 21	3.875	0.7208	3.115	2.0388	2.41	8.5	D_7-D_{30}: 0.889	
	Day 30	4.7763	1.0281	4.035	2.9079	1.28	10	D_{14}-D_{21}: 0.8438	
Statistical significance (p) between groups		D_7 (I-II): 0.065		D_{14} (I-II): 0.2923		D_{21} (I-II): 0.1505		D_{30} (I-II): **0.0031**	
		D_7 (I-III): 0.1049		D_{14} (I-III): 0.0618		D_{21} (I-III): 0.065		D_{30} (I-III): 0.0681	
		D_7 (II-III): 0.1423		D_{14} (II-III): 0.0902		D_{21} (II-III): 0.7209		D_{30} (II-III): 0.7945	

Table VI. Comparative analysis of CP values (measured in mg%) at the studied moments and statistical significance.

Group	Moment	Mean	SE	Median	SD	Min.	Max.	*Statistical significance (p) between moments*	
I	**Day 7**	50.5175	1.8926	50.52	5.3531	40.4	58.7	D_7-D_{14}: 0.8143	D_{14}-D_{30}: **1.26 x 10^{-05}**
	Day 14	51.2175	1.7291	51.38	4.8906	44.2	59.1	D_7-D_{21}: **0.0009**	D_{21}-D_{30}: **0.044**
	Day 21	61.0688	1.6769	60.625	4.7430	55.3	68.3	D_7-D_{30}: **0.0006**	
	Day 30	69.0125	2.3784	69.1	6.7272	60.2	77.1	D_{14}-D_{21}: **0.0139**	
II	**Day 7**	48.1325	1.2268	47.955	3.4699	43.8	53.9	D_7-D_{14}: **0.0027**	D_{14}-D_{30}:**0.0027**
	Day 14	59.4250	3.1918	59.4	9.0279	46.9	73.6	D_7-D_{21}: **0.0078**	D_{21}-D_{30}: 0.4609
	Day 21	65.7625	3.0306	69	8.5717	54.6	75.4	D_7-D_{30}:**2.67 x 10^{-06}**	
	Day 30	69.1688	1.9898	67.425	5.6281	63.4	79.4	D_{14}-D_{21}: **0.0469**	
III	**Day 7**	60.5825	2.1894	60.58	6.1926	51.3	68.4	D_7-D_{14}: 0.5834	D_{14}-D_{30}:**0.0118**
	Day 14	57.3675	4.7763	54	13.5093	44.8	81	D_7-D_{21}: **0.0214**	D_{21}-D_{30}:**0.0341**
	Day 21	65.1813	2.5784	63.15	7.2929	57.5	78.65	D_7-D_{30}: **0.0041**	
	Day 30	72.6188	1.6954	73.2	4.7954	64.5	78.65	D_{14}-D_{21}: 0.1775	
Statistical significance (p) between groups		D_7 (I-II): 0.3111		D_{14} (I-II): **0.045**		D_{21} (I-II): 0.3282		D_{30} (I-II): 0.9605	
		D_7 (I-III): **0.0037**		D_{14} (I-III): 0.2568		D_{21} (I-III): 0.206		D_{30} (I-III): 0.2388	
		D_7 (II-III): **0.0004**		D_{14} (II-III): 0.7264		D_{21} (II-III): 0.9591		D_{30} (II-III): 0.2081	

Although considering all studied groups on day 7, there should have been statistically significant differences between at least two of these, the statistical analysis of values for unpaired samples did not reveal these statistically significant differences.

f. Ceruloplasmin

The statistical analysis of *CP* values (Table VI), *considering all studied moments,* evidenced highly statistically significant differences between at least two of the studied moments in groups I ($p=2.96 \times 10^{-7}$) and II ($p=0.0003$) and very statistically significant differences between at least two of the studied moments in group III ($p=0.0087$).

The statistical analysis of *CP* values *for paired samples* showed:

- in group I – highly statistically significant differences between days 7-21, 7-30 and 14-30 ($p<0.001$) and statistically significant differences between days 14-21 and 21-30 ($p<0.05$);
- in group II – highly statistically significant differences between days 7-30 ($p<0.001$), very statistically significant differences between days 7-14, 7-21 and 14-30 ($p<0.01$) and statistically significant differences between days 14-21 ($p<0.05$);
- in group III – very statistically significant differences between days 7-30 ($p<0.01$) and statistically significant differences between days 7-21, 14-30 and 21-30 ($p<0.05$).

The statistical analysis of *CP* values**,** *considering all groups at a certain moment,* revealed highly statistically significant differences between at least two of the groups on day 7 ($p=0.00019$). On days 14, 21 and 30, no statistically significant differences between any of the groups were found ($p=0.2418$, $p=0.417$, and $p=0.3854$, respectively).

Although in some cases, considering all studied groups, no statistically significant differences between these could be found, the statistical analysis of *CP* values *for unpaired samples* showed:

- on day 7 – highly statistically significant differences between groups II-III ($p<0.001$), very statistically significant differences between groups I-III ($p<0.01$);
- on day 14 – statistically significant differences between groups I-II ($p<0.05$).

Discussion

The balance between ROS and NO has played an important role in the development of atherosclerosis in patients with peripheral arterial disease.

Cell and gene therapy together with antioxidative pharmacological therapy can be useful in recovering the function of the endothelium and preventing the development of atherosclerosis in patients with critical ischemia [22].

Critical ischemia is characterised by an increased production of all the ROS [23].

In our study we evaluated the effect of the therapy with Sildenafil and Donepezil on the markers of OS and endogeneous AO.

When analysing the groups we noticed the decrease of MDA in the group treated with Donepezil compared to the group that had no treatment and the group with Sildenafil and a significant increase of MDA in the 14th day of treatment. Another marker of OS, GSSG shows a significant decrease in the Sildenafil, Donepezil group compared to the group with ligature and no treatment. Aydin et al. [24] had the same results when administering another vasodilator, levosimendan, which has a different mechanism of action by opening ATP dependent potassium channels (K_{ATP}) in the smooth muscle.

According to some theories, Sildenafil is supposed to use the same mechanism of action [25]. However, the decrease in the MDA blood level seen in the Donepezil group can be interpreted through the different mechanism of action: Donepezil lengthens the dilating action of Ach [14] on an intact endothelium; this is true for vessels of neoformation but not for injured vessels.

The administration of PDE5 inhibitor and Sildenafil, decreasing the serum level of GSSG but also decreasing the GSH/GSSG ratio, significantly decreased by the 30th day, in the group with Sildenafil, compared to the group with no treatment.

The 14th day appears to display the highest OS level both in the group with Sildenafil and in the Donepezil therapy group, proved by high levels of stress markers, MDA and GSSG that are decreasing on the 21st day. There are also low levels of AO (GSH) which can be explained by their use in the effort of neutralizing free radicals.

These results are in agreement with the studies of Bivalacqua [26] that demonstrate reduced levels of ROS under the effect of Sildenafil associated with an improvement in endothelial function supported in our study by the increase in e NOS level.

A recent study [27] demonstrates the same effect of reduced oxidative stress by using another PDE5 inhibitor, tadalafil, the effect being reached by lowering MDA level and raising SOD level after 8 weeks of treatment.

Actually PDE5 inhibitors are considered to play an important role in reducing OS in other organs as well. Sildenafil decreases OS and protects the heart in patients with left ventricular hypertrophy and congestive heart failure. Also, at the level of pulmonary artery, Sildenafil diminishes OS, neutralizes the inflammation and remodeling induced by smoking [28]

Our results are in accordance with the literature data regarding the GSH/GSSG couple, which is the most important intracellular redox buffer and the redox indicator of the cellular environment [29,30].

The increase of CP, an extracellular AO, shows the intervention of defence mechanisms in OS, associated with ischemia and reperfusion. The fact that CP increases in all

groups and at all times is probably due to its intervention on acute phase reactant in the inflammatory processes of chronic ischemia [31].

Our results show that Sildenafil and Donepezil administration might contribute to the acute increase of blood flow in ischemic tissues, the increase of ischemia-induced angiogenesis and of endothelial cell proliferation in ischemic areas, as previous shown by other studies [32], processes that might also be stimulated by the low ROS production after 14 days. The beneficial effects of ROS occur at low or moderate ROS concentrations [33,34].

Conclusions

1. The administration of Sildenafil and Donepezil has favorable effects in reducing OS in experimentally induced CLLI.

2. Sildenafil and Donepezil administration stimulates extracellular AO defence on account of CP.

3. Sildenafil and Donepezil administration influences intracellular redox homeostasis on account of the GSH/GSSG couple, the major redox buffer in the body.

References

1. Karmazyn M. Ischemic and reperfusion injury in the heart: cellular mechanisms and pharmacologic interventions. Can J Physiol Pharmacol, 1991; 69:719-730.
2. Turrens JF, Beconi M, Barilla J, et al. Mitochondrial generation of oxygen radicals during reoxygenation of ischemic tissues. Free Radic Res Commun, 1991; 12-13(Pt 2):681-689.
3. Hiatt WR, Brass EP. Pathophysiology in perypheral artery disease, intermittent claudication and critical limb ischemia in Vascular Medicine: A Companion to Braunwald's Heart Disease (Second Edition), 2013; 223-230.
4. Du F, Zhou J, Gong R, et al. Endothelial progenitor cells in atherosclerosis. Front Biosci, 2012; 17: 2327-2349.
5. Yu J, deMuinck ED, Zhuang Z, et al. Endothelial nitric oxide synthase is critical for ischemic remodeling, mural cell recruitment, and blood flow reserve. PNAS, 2005; 102-31: 10999-11004
6. Contreras DL, Robles HV, Romo E, Rios A, Escalante B. The role of nitric oxide in the post- ischemic revascularisation process. Pharmacol Ther, 2006; 112:553-563.
7. Cooke JP. NO and atherosclerosis. Atherosclerosis, 2003; 4:53-60.
8. Sessa WC. Molecular control of blood flow and angiogenesis: role of nitric oxide. J Thromb Haemost, 2009; 7:35-37.
9. Lekas M, Lekas P, Latter D, Kutryk M, Stewart D. Growth factor induced therapeutic neovascularisation for ischemic vascular disease: time for a re-evaluation? Curr Opin Cardiol, 2006; 21:376-384.
10. Contreras DL, Robles HV, Romo E, Rios A, Escalante B. The role of nitric oxide in the post- ischemic revascularisation process. Pharmacol Ther, 2006; 112:553-563.
11. Elrod JW, Duranski MR, Langston W, et al. eNOS gene therapy exacerbates hepatic ischemia-reperfuzion injury in diabetes: a role for eNOS uncoupling. Circ Res, 2006; 99:78-85.
12. Senthilkumar A, Smith RD, Khitha J, et al. Sildenafil promotes ischemia-induced angiogenesis through a PKG dependent pathway. Arterioscler Thromb Vasc Biol, 2007; 27:1947-1954.
13. Kuwabara M, Kakiuma Y, Ando M, et al. Nitric oxide stimulates vascular endotelial growth factor production in cardiomyocytes involved in angiogenesis. J Physiol Sci, 2006; 56:95-101.
14. Kakinuma Y, Furihata M, Akiyama T, et al. Donepezil, an acetylcholinesterase inhibitor against Alzheimer's dementia, promotes angiogenesis in an ischemic hindlimb model. J Mol Cell Cardiol, 2010; 48:680-693.
15. Walker DK, Ackland MJ, James GC, et al. Pharmacokinetics and metabolism of sildenafil in mouse, rat, rabbit, dog and man. Xenobiotica, 1999; 29:297-310.
16. Colleran PN, Zeyi LI, Yang HT, Laughlin MH, Terjung RL. Vasoresponsiveness of collateral vessels in the rat hindlimb: influence of training. J Physiol, 2010; 588:1293-1307.
17. Yang HT, Ren J, Laughlin MH, Terjung RL. Prior exercise training NO-dependent increases in collateral blood flow after acute arterial occlusion. M J Physiol Heart Circ Physiol, 2002; 282:H31-H310.
18. Conti M, Moran PC, Levillain P, et al. Improved fluorimetric determination of malondialdehyde. Clin. Chem, 1991; 37:1273-1275.
19. Reznick AZ, Packer L. Oxidative damage to proteins: spectrophotometric method for carbonyl assay. Methods in enzymology, 1994; 233:357-363.
20. Hu ML. Measurement of protein thiol groups and glutathion in plasma. Meth. Enzymol, 1994; 233:380-383.
21. Ravin HA. Determinarea ceruloplasminei. În Manta C, Cucuianu M, Benga G, Hodarnau A. Metode biochimice în laboratorul clinic. Ed. Dacia, Cluj-Napoca, 1976; 330-331.
22. Higashi Y, Nishioka K, Umemura T, Chayama K, Yashizumi M. Oxidative stress, endothelial function and angiogenesis induced by cell therapy and gene therapy. Curr Pharm Biotechnol, 2006; 7:109-116.
23. Dopheide JF, Doppler C, Scheer M, et al. Critical limb ischaemia is characterised by an increased production of whole blood reactive oxygen species and expression of TREM-1 on neutrophils. Atherosclerosis, 2013; 229:396-403.
24. Aydin C, Ay Y, Base H, et al. Analysis of the influences of short-term levosimendan exposure on oxidant/antioxidant status and trace-element levels in the physiological status of the thoracic aorta of rats. J Membr Biol, 2012; 245:827-832.
25. Taira N. Nicorandil as a hybrid between nitrates and potassium channel activators. Am J Cardiol, 1989; 63:18J-24J.
26. Bivalacqua TJ, Sussan TE, Gebska MA, et al. Sildenafil inhibits superoxide formation and prevents endothelial dysfunction in a mouse model of secondhand smoke induced erectile dysfunction. J Urol, 2009; 181:899-906.
27. Chen Y, Li XX, Lin HC, et al. The effects of long-term administration of tadalafil on STZ-induced diabetic rats with erectile dysfunction via a local antioxidative mechanism. Asian J Androl, 2012; 14:616-620.
28. Milara J, Juan G, Ortiz JL, et al. Cigarette smoke-induced pulmonary endothelial dysfunction is partially suppressed by sildenafil. Eur J Pharm Sci, 2010; 39: 363-372.
29. Dröge W. Aging-related changes in the thiol/disulfide redox state: implications for the use of thiol antioxidants. Exp Gerontol, 2002; 37:1333-1345.
30. Schafer FQ, Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radic Biol Med, 2001; 30:1191-212.

31. Giurgea N, Constantinescu MI, Stanciu R. Ceruloplasmin - acute phase reactant or endogenous antioxidant? The case of cardiovascular disease. Med Sci Monit, 2005; 11: RA48-RA51.
32. Kumar D, Branch BG, Pattillo CB, et al. Chronic sodium nitrite therapy augments ischemia-induced angiogenesis and arteriogenesis. Proc Natl Acad Sci USA, 2008; 105:7540-7545.
33. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol, 2007; 39:44-84.
34. Tache S. Stresul oxidativ. În Dejica D (sub red). Stresul oxidativ în bolile interne. Ed. Casa Cărții de Știință, Cluj-Napoca, 2000; 2:77-100.