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IN VITRO STUDIES REGARDING THE ANTIOXIDANT EFFECTS OF PROCAINE, GEROVITAL H3 AND ASLAVITAL

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Gerovital H3 (GH3) and Aslavital are Romanian procaine based drugs used in the prophylaxis of aging for over 40 years. By the use of *in vitro* experimental models, the main purpose of the present study was to evaluate the antioxidant effects of these anti-aging products with regard to the superoxide radical generation in an enzymatic system, xanthine - xanthine oxidase - INT [2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride], as well as with regard to the susceptibility of human low density lipoprotein to oxidation (oxLDL), corresponding to conjugated dienes formation. At 10 mM in reference to procaine concentration**,** the maximum of inhibition was exerted by Aslavital (88%) followed by GH3 (62%), whereas procaine hydrochloride had a slight inhibitory effect upon the INT reduction. We pointed out a significant inhibition of Cu^{2+}- induced oxLDL after 20 and 60 minutes in the presence of Aslavital at all the concentrations tested (0.1; 0.5 and 1 mM, related to procaine final concentration in the reaction system), while GH3 exerted a significant decrease in oxLDL only at the highest concentration (1mM procaine).

INTRODUCTION

Gerovital H3 and Aslavital are original procaine based anti-aging products developed and studied by Prof. Dr. Ana Aslan in the sixties and seventies, particularly designed to slow the aging process. These two Romanian drugs interfere with the mechanisms of aging at cellular and molecular levels as well as with common mechanisms of chronic degenerative age-related diseases.[1]

Gerovital H3 (GH3) used in the prophylaxis of aging for over 40 years contains procaine hydrochloride (2%) as its active ingredient, benzoic acid (0.12%), disodium phosphate (0.01%), potassium metabisulphite (0.10%), and has a pH 3.3. Aslavital contains procaine hydrochloride (2%), potassium metabisulphite (0.10%), benzoic acid (0.12%), disodium phosphate, L- glutamic acid (potassium salt) (1.2%), and has a pH 3.3.[2]

As a part of the growing interest in therapeutic strategies for anti-aging and disease prevention, antioxidants have become a major area of focus. Antioxidant testing has become a cornerstone of the pharmaceutical products industry.

Under normal circumstances, there are large numbers of antioxidants, synthesized within the body or taken from the diet, that form a natural defense against free-radical induced damage. Free radicals are species containing one or more unpaired electrons and include unstable oxygen radicals such as superoxide radical ($O_2^{\cdot -}$) and hydroxyl radical (HO•), and nonradical molecules like hydrogen peroxide (H_2O_2). These reactive oxygen species, which are continuously generated as byproducts of normal aerobic metabolism, can also be produced to a greater extent under stress and pathological conditions, as well as taken up from the external environment.[3]

Antioxidants react with the free radicals to neutralize their effect by donating electrons, thereby forming much less reactive radicals or stable products. The imbalance between protective antioxidants and damaging free radicals defines the oxidative stress. In the group of antioxidants are included enzymes, such as superoxide dismutase, catalase and glutathione peroxidase, which mainly act within the intracellular compartments, and low-molecular-mass scavengers (vitamins C, A, E, carotenoids, albumin, uric acid, and total bilirubin)

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in the circulation, that protect lipoproteins from oxidative modification in the extracellular fluid.[4]

By the use of *in vitro* experimental models the major purpose of the present study was to evaluate the antioxidant effects of procaine, GH3 and Aslavital with regard to the superoxide radical generation in an enzymatic system, xanthine-xanthine oxidase, as well as with regard to the susceptibility of human low density lipoprotein to oxidation (oxLDL).

EXPERIMENTAL

Superoxide generation by the xanthine-xanthine oxidase (XO) system

Superoxide anions generated by the xanthine-XO system were assayed by the reduction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to a red formazan dye. The reaction mixture comprised a solution of 100 μM xanthine, 0.07 U/mL xanthine oxidase and 60 μM INT in a total volume of 1 mL, in 0.1 M phosphate buffer at pH 7.4.[5] This mixture was incubated at 25 ^{0}C for 10 min and the absorbance was continuously recorded for 5 minutes at 505 nm against a blank which did not contain the enzyme, on Perkin Elmer Spectrophotometer, Lambda Bio-10. Only the linear part of the reaction curve (3 minutes) was used in the calculations and the results were expressed as Δ absorbance at 505 nm/min. In order to evaluate the inhibitory effect of procaine hydrochloride, Gerovital H3 and Aslavital on superoxide generation, various concentrations of these products, in reference to procaine concentrations, ranging between 1 and 10 mM, were added to this enzymatic system. Cu/Zn-Superoxide dismutase (SOD) was used as a positive control for this inhibitory effect.

LDL isolation and oxidation

The native LDL fraction was isolated from fresh human plasma obtained from 5 normolipidemic, apparently healthy volunteers (women aged between 35 and 65 years), using a single vertical discontinuous density gradient ultracentrifugation.[6] None of the subjects were taking any dietary antioxidants. The kinetics of Cu^{2+}-induced lipoprotein oxidation, corresponding to conjugated dienes (CD) formation, was evaluated by monitoring the changes in absorbance at 234 nm (37°C), on a Kontron double beam spectrophotometer (Uvikon 933) equipped with a 10 position automatic sample changer. To determine *in vitro* LDL oxidizability, copper-catalyzed oxidation was performed with 50 μg /mL LDL protein and 5μM $CuSO_4$ for a three-hour period, at 37°C. CD formation was recorded every 2 minutes and the kinetic parameters of LDL oxidation were determined from the time-course curves. Data are expressed as the increase in absorbance over baseline (ΔA234). From these data, the formation of CD was calculated as conjugated dienes equivalent content (nmoles CD/mg protein) during 3 hours, after Cu^{2+}-induced LDL oxidation, using the CD molar extinction coefficient at 234 nm (29500 $molesL^{-1}cm^{-1}$). Different procaine, GH3 and Aslavital concentrations (0.1; 0.5 and 1 mM related to the procaine final concentration) were added in the oxidation medium, in order to assess their effects. In control samples, 20 μM butylated hydroxytoluene (BHT)- a synthetic antioxidant, and 100 μM EDTA, were used to inhibit the lipoprotein oxidation.

Statistical analysis

Data from *in vitro* experiments were obtained from five independent experiments. Results were expressed as mean values ± standard deviation (SD). Statistical analysis was performed in order to assess differences between experimental conditions and between biochemical parameters, such as INT reduction and CD formation, in the presence or in the absence of GH3, Aslavital and procaine hydrochloride, by using unpaired, two-tailed Student's *t* test, with Microsoft EXCEL for Windows XP. A difference was considered statistically significant when $p< 0.05$.

RESULTS

Effects of procaine, GH3 and Aslavital on superoxide radical anion generation

The INT salts reduction by $O_2^{\cdot -}$ produced enzymatically in the xanthine-xanthine oxidase system, took place according to the following reactions:

$$\text{Xanthine} + O_2 \xrightarrow{\text{XO}} \text{Uric acid} + O_2^{\cdot -} \quad (1)$$

$$\text{INT} + O_2^{\cdot -} \longrightarrow \text{formazans} \quad (2)$$

The enzyme superoxide dismutase (SOD) catalyses the superoxide radical anion dismutation:

$$O_2^{\cdot -} + O_2^{\cdot -} \xrightarrow{\text{SOD}} H_2O_2 + O_2 \quad (3)$$

The xanthine-xanthine oxidase system is the enzymatic system which is often used as a generator of superoxide radicals.[5] Xanthine oxidase (XO) catalyzes the oxidation of xanthine in the presence of molecular oxygen to yield uric acid and $O_2^{\cdot -}$ as reaction products (reaction 1). The

superoxide anion formed reduces the INT salts to formazans (reaction 2).

INT reduction in the superoxide generating system xanthine-xanthine oxidase was performed in the absence and in the presence of increasing concentrations of GH3, Aslavital and procaine hydrochloride, compared to SOD- the antioxidant enzyme that detoxifies superoxide at physiological level (reaction 3).

Our results show that GH3 and Aslavital inhibited the superoxide radical-mediated INT reduction, in different degrees depending on their concentrations in the reaction mixture (Table 1).

Table 1

The inhibitory effect of different concentrations of Gerovital H3, Aslavital, procaine and superoxide dismutase (SOD) on INT reduction (Δ absorbance/Δ time) by superoxide, in the xanthine-xanthine oxidase generating system

Sample	Concentrations	INT reduction (Δ absorbance at 505 nm/min)	Inhibition (%) of INT reduction
Control (Xanthine - Xanthine oxidase)	-	0.23	-
SOD	0.337 U/ml	0.10	56
	0.675 U/ml	0.08	66
	1.35 U/ml	0.05	77
	2.7 U/ml	0.03	87
	5.4 U/ml	0.02	92
Gerovital H3	1 mM	0.22	5
	2 mM	0.20	12
	3 mM	0.18	21
	5 mM	0.16	31
	7 mM	0.13	41
	10 mM	0.09	62
Aslavital	1 mM	0.19	16
	2 mM	0.18	22
	3 mM	0.14	40
	5 mM	0.11	52
	7 mM	0.06	74
	10 mM	0.03	88
Procaine hydrochloride	1 mM	0.23	-
	2 mM		
	3 mM		
	5 mM		
	7 mM		
	10 mM	0.21	5

Each data value represents the means of three experiments.

The maximum inhibition percentage of INT reduction of 92% was obtained by adding 5.4 U/ml SOD in the system. Also, the percentage inhibition on INT reduction to formazans increased with increasing GH3 and Aslavital – related procaine concentration in the reaction system. At all the tested concentrations (1, 2, 3, 5, 7 and 10 mM), the maximum of inhibition was exerted by Aslavital (88% at 10 mM) followed by GH3 (62%), whereas procaine had a slight inhibitory effect at its maximal concentration in the system.

Effects of procaine, GH3 and Aslavital on LDL oxidation

Peroxidation of polyunsaturated fatty acids side chains of LDL leads to the formation of a conjugated diene (CD), with a characteristic UV absorption maximum at 234 nm. Evaluation of CD is one of the most widely used methods for monitoring LDL oxidation *in vitro*. The basal oxidative state of LDL can be assessed as the baseline absorbance of the CD assay before the addition of copper. Comparison of time-course curves of LDL oxidation induced by a pro-oxidant transition metal ion, Cu^{2+}, in the presence and absence of different concentrations of GH3, Aslavital and procaine hydrochloride is presented in Figure 2.

The inhibitory effect of Aslavital, GH3 and procaine on the CD formation is exerted in the first segment of the time-course oxidation and is reduced after 120 minutes. The addition of $CuSO_4$ on native LDL increased the level of the CD and

was significantly different from control samples, incubated with BHT and EDTA. The results regarding the effect of GH3, Aslavital and procaine hydrochloride on Cu^{2+}- induced LDL oxidation at different time points, are presented in Table 2.

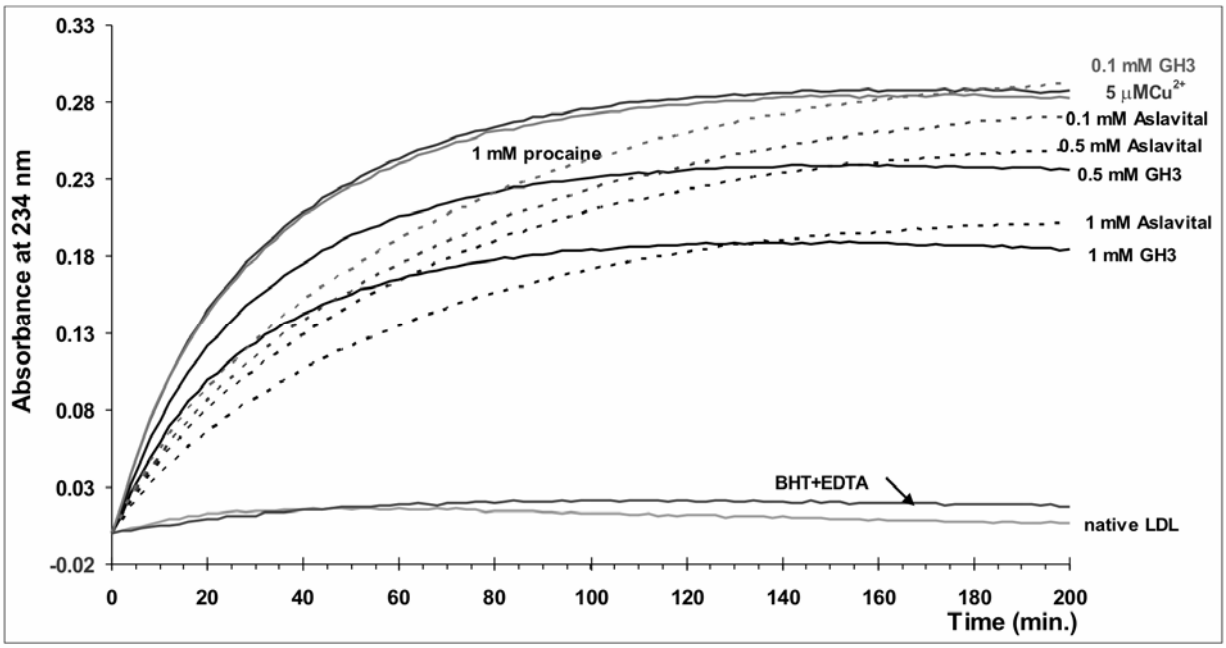

Fig. 2 – Representative spectrophotometric recording illustrating the effect of different concentrations of GH3, Aslavital and procaine hydrochloride on the formation of conjugated dienes in Cu^{2+}- induced LDL oxidation.

Table 2

Kinetics of the formation of conjugated dienes during Cu^{2+}- induced oxidation of human low-density lipoprotein (oxLDL), in the presence of different concentrations of Gerovital H3, Aslavital and procaine hydrochloride

Sample conjugated dienes (nmoles/ mg LDL)	**20 minutes**	**60 minutes**	**120 minutes**	**180 minutes**
Native LDL	8.5 ± 1.1	10.6 ± 0.8	7.9 ± 0.6	5.3 ± 0.4
LDL + 5 μM Cu^{2+}	98.2 ± 8.2	164.9 ± 17.2	191.7 ± 18.7	195.0 ± 18.1
LDL + 5 μM Cu^{2+}				
+ 1.0 mM GH3	67.4 ± 4.8**	111.5 ± 9.5**	127.2 ± 10.8**	126.8 ± 9.7**
+ 0.5 mM GH3	82.4 ± 6.3*	139.6 ± 12.2	160.4 ± 15.2	161.5 ± 14.3
+ 0.1 mM GH3	96.6 ± 9.3	163.1 ± 15.4	188.7 ± 14.9	193.2 ± 17.2
+ 1.0 mM Aslavital	44.9 ± 3.7***	91.1 ± 8.7***	124.3 ± 11.3**	135.4 ± 12.2**
+ 0.5 mM Aslavital	54.3 ± 3.8***	111.4 ± 10.2**	152.5 ± 13.7*	167.8 ± 12.6
+ 0.1 mM Aslavital	58.5 ± 5.5***	119.0 ± 10.8**	162.8 ± 14.9	181.1 ± 16.1
+ 1.0 mM procaine hydrochloride	64.0 ± 5.0**	129.5 ± 11.1*	176.2 ± 15.1	195.2 ± 17.5
LDL + 5 μM Cu^{2+} + BHT + EDTA	5.9 ± 0.5	12.6 ± 1.3	14.3 ± 3.5	12.5 ± 2.5

Each data value represents the means of five experiments.
Differences between experimental conditions, namely Cu^{2+}- induced LDL oxidation *versus* Cu^{2+}- induced LDL oxidation in the presence of GH3, Aslavital and procaine hydrochloride, were assessed by Student's *t*-test.
* Values of CD significantly lower, generated after addition of GH3, Aslavital or procaine in the incubation medium, measured at different time points: 20, 60, 120 and 180 minutes.
*** $p<0.001$; ** $p<0.01$; * $p<0.05$.

We pointed out a significant inhibition of Cu^{2+}-induced LDL oxidation after 20 and 60 minutes in the presence of Aslavital at all the concentrations tested, while GH3 exerted a significant decrease in LDL susceptibility to oxidation only at the highest concentration (1mM). After 180 minutes from LDL-induced oxidation, only 1 mM GH3 or Aslavital significantly inhibited the CD formation. The

concentration of 1 mM procaine in the oxidation medium significantly inhibited LDL oxidation only at 20 and 60 minutes after its inducement.

DISCUSSION

It has been established that oxidative stress plays an important role in aging, and various diseases such as: arteriosclerosis, ischemia and reperfusion tissue injury, in inflammation and immunological processes, in toxic injury as well as in carcinogenesis. In addition, the mounting genetic evidence that links oxidative stress to aging is discussed, as well as the potential challenges and benefits associated with the development of anti-aging interventions and therapies.[4]

Researches with regard to the antioxidant action of the anti-aging products GH3, Aslavital and procaine, were carried out at the National Institute of Gerontology and Geriatrics in the context of asserting the free radicals theory in aging and associated pathology, elaborated by D. Harman, in 1956.[8]

It has been proposed that toxic O_2 metabolites generated by xanthine oxidase contribute to the development of injury seen during reperfusion of a variety of ischemic tissues.[3] Therefore, we examined whether procaine based pharmaceutical preparations inhibit the superoxide ($O_2^{\cdot -}$) enzymatic generation by the xanthine-xanthine oxidase (XO). We found that Aslavital and GH3 inhibited the INT reduction in a dose-dependent manner, at degrees comparable to those exerted by SOD. It is possible, however, for these procaine based products to have more than one way of affecting xanthine-xanthine oxidase reactions: as inhibitors of xanthine oxidase, and as scavengers of the superoxide anion, but most probably in both ways.

Our results are in accordance to those of Rusu and Lupeanu[9] with respect to the inhibitory actions exerted by these anti-ageing products as well as some of their ingredients, such as potassium metabisulphite and potassium glutamate, on the superoxide radical generation in Nishikimi's non-enzymatic system which contains reduced nicotinamide adenine dinucleotide phosphate (NADH) - phenazinmetosulfate (PMS) - nitroblue tetrazolium (NBT).

Increased $O_2^{\cdot -}$ production by several potential mechanisms, including enzymatic sources such as xanthine oxidase and NAD(P)H oxidase, has been implicated in endothelial dysfunction and formation of oxidized lipids (oxLDL) as a key factor in the pathogenesis of atherosclerosis.[10] The initiation of LDL lipid peroxidation is induced by the superoxide or by hydroxyl radicals.[6] The first stage of lipid peroxidation consists in the molecular rearrangement of the double bonds in polyunsaturated fatty acids residues of lipids, which leads to conjugated dienes (CD) formation and conversion of CD in hydroperoxides.[7] There are numerous evidences that antioxidants may prevent atherogenesis by inhibiting lipid peroxidation.[4,11] Hence, we assessed the LDL oxidation by monitoring the formation of CD in LDL isolated from human plasma, in the presence of Gerovital H3, Aslavital and procaine hydrochloride. Results clearly show that GH3 and Aslavital significantly decreased the susceptibility to *in vitro* copper induced oxidation of LDL. Point-by-point analysis between time-course oxidation curves showed significant differences in the effects of all three compounds.

Previous studies regarding these compounds' inhibitory role on lipid peroxidation suggest that procaine can exert antioxidant effects through a mechanism of binding to the microsomal and mitochondrial membrane structures, the latter thus becoming less susceptible to the free radical reactions.[12,13] Neither the possibility of a mechanism by direct trapping of free radicals, nor that of increases in endogenous antioxidant concentrations can be ruled out, especially as significant increases in serum total antioxidant capacity were pointed out in patients who received treatment with the two anti-aging products.[14]

CONCLUSIONS

Results obtained in this study confirm the role of the Roumanian procaine based products as protective agents against LDL oxidation as well as inhibitory agents on $O_2^{\cdot -}$ generation, and complete data that could explain, certainly to a partial extent, the anti-atherogenic effects of Aslavital and Gerovital H3, proved from a clinical standpoint in longitudinal studies. Further investigations are needed to establish the exact mechanism of action of these antioxidant therapeutic agents.

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