ANTIOXIDANTS PROLONG SURVIVAL OF LYMPHOCYTES

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SUMMARY: The trace metals (antioxidants) were studied to elucidate their effects to prolong lymphocyte survival by preventing programmed cell death (apoptosis), these trace metals; $ZnCl_2$ ($4.77x10^{-5}$ M), $CuSO_4$ ($15x10^{-6}$ M) were added to the lymphocyte cultures on the third day in RPMI media. H_2O_2 was used to promote apoptosis.

Apoptosis was assessed morphologically (by phase contrast microscopy and DNA quantitation) and biochemically (MDA production, thiol, and selenium consumption). In this study which includes 31 samples in the control group and 31 in the test group, lymphocyte apoptosis was found to be associated with generation of free radicals and that the antioxidants significantly inhibit apoptotic process. It was noted that H_2O_2 enormously accelerates apoptosis.

Key Words: Apoptosis, antioxidants, trace metals, free radicals.

INTRODUCTION

The term *'free radicals'* or reactive oxygen species (ROS) refers to any species that contains one or more unpaired electron(s), like singlet oxygen, superoxide anion ($O^{\cdot -}_2$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$). The latter is the most reactive oxygen species (1).

The *free radicals* are able to cause many changes at the cellular level. They are involved in physiological conditions as well as pathological cellular events. On the other hand, they can reduce the oxidation of the unsaturated fatty acids (lipid peroxidation) that may explain at least in part pathogenesis of many diseases (2).

Apoptosis or programmed cell death (PCD) is one of the physiological phenomena in which ROS play a major role (3,4). In this respect ROS are suggested to cause caspases activation and cleavage (caspases 3 and 6), that trigger and initiate the sequence of PCD (4). Hydrogen peroxide is one of the ROS that are able to increase CD95 ligand expression that leads to sequences of caspase activation (5). ROS are also important in processes that lead to the leakage of cytochrome c from mitochondria, which enhance apoptotic process (4).

Many trace metals are known to play a role in the mechanism and regulation of PCD through promotion or amelioration of the different steps of apoptosis. Some of the trace metals studied in this direction are zinc, copper, and selenium.

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Table 1: Effects of H_2O_2 and trace metals on morphological staging of lymphocyte apoptosis.

N=31	Time	Total non-viable cells	Stage I	Stage II	Stage III
			No	No	No
Blank 37°C	0 h	4.22 ± 0.51	4.22 ± 5.1	0	0
	24 h	40.72 ± 7.11	4.33 ± 0.04	13.4 ± 3.3	23.01± 9.88
	72 h	100 ± 0.0	2.11 ± 0.31	40.91± 9.99	58.21± 13.01
H_2O_2	10 min	11.5 ± 2.66	8.66 ± 0.77	3.5 ± 0.58	0
	24 h	100 ± 0.0	15.13 ± 2.1	35.11 ± 10.1	50.45 ± 9.99
Copper	24 h	35.33 ± 6.2	5.11 ± 0.66	12.23 ± 6.21	18.88 ± 3.46
	72 h	69.11 ± 11.3	4.31 ± 0.41	25.55 ± 11.10	40.82 ± 5.66
Zinc	24 h	29.21 ± 5.21	5.61 ± 0.33	10.11 ± 1.89	14.87 ± 3.34
	72 h	57.31 ± 10.11	5.56 ± 0.41	18.25 ± 2.01	34.52 ± 8.99

Results are expressed as mean ± SE of the mean.

Zinc offers an increment in cellular resistance against PCD via different mechanisms (5). It is regarded as inhibitor for caspase 3 and 6 activations. Zinc can also inhibit Ca/Mg dependent endonuclease and increases BCl_2/bax family ratio. Moreover zinc ameliorates PCD processes through its antioxidant effect. Zinc can stabilize sulphydryl functioning protein (6) and reduces hydroxyl free radical generation ($OH^{\cdot}$, O_2^{-}) from H_2O_2 (1). It is also involved in the scavenger ability of superoxide dismutase enzyme, particularly Cu/Zn dismutase enzyme (7).

Copper ion is a component of the enzyme Cu/Zn SOD which regulates the intracellular concentration of superoxide anion ($O^{\cdot -}_2$), by converting it into H_2O_2 (2). Copper is the important part of mitochondrial enzyme Cytochrome c oxidase (8).

Selenium acts through the selenoenzyme-glutathione peroxidase (2). Deficiency in selenium level results in decrease of glutathione peroxidase activity and thereby leads to susceptibility of tissues to injury by reactive oxygen species (2).

These trace metals can protect cells from death by apoptotic process. Apoptosis is a distinct morphological type of cell death, which requires gene expression, mediated by cell stimulation. The cell undergoes an internally active transition from intact, metabolically active state into a number of shrunken remnants retaining their membrane-bound integrity (3). In this process the biologically active cellular components are surrounded by cell membrane throughout the process of apoptosis that is why these cells are eliminated without inflammation (9). Apoptosis is associated with the generation of free radicals that can be regarded as a method for diagnosis of apoptosis (10).

MATERIALS AND METHODS

This study was designed as an *in vitro* experiment on the effect of trace metals (antioxidants) against lymphocyte apoptosis.

Some physiological factors affecting apoptosis like:

i. Stimulation of apoptosis by H_2O_2 10^{-8} M.

ii. Inhibition of apoptosis by antioxidants as $ZnCl_2$ (4.77×10^{-5} M) and $CuSO_4$ (15×10^{-6} M).

Peripheral blood lymphocytes were separated by Ficoll 400 (Pharmacia fine chemicals). Lymphocytes cultured in RPMI media 1640 (Flow laboratories) containing L-glutamine, streptomycin, penicillin, and FCS 10% were added to the culture media. The number of experiments conducted was 3, each group consists of 31 subjects. These were as follows:

1. Control group (lymphocytes cultured at 37°C without manipulation).

2. The addition of pro-oxidant to the incubation media (H_2O_2 10^{-8} M).

3. The addition of antioxidant to the incubation media, these are $ZnCl_2$ and $CuSO_4$.

These three groups were studied at time zero: the time of separation of lymphocytes, after 24 hours of incubation and after 72 hours of incubation except for second group the examination was at time zero, then after 10 minutes and 24 hours of incubations.

Table 2: Effect of H_2O_2 on the cellular and biochemical changes related to lymphocyte apoptosis.

N=31	Time			Rate of changes (b)			Status
	0	10 min	24 h	B1	B2	Bt	p value
TBE	3.36 ± 0.54	11.66 ± 2.40	100 ± 0	51.87	3.7	3.86	<0.05
DNA	207.98 ± 18.96	190.76 ± 7.38	140.27 ± 8.35	107.6	2.11	2.47	<0.05
MDA nmol/ml	2.96 ± 0.8	3.02 ± 0.73	3.89 ± 0.64	0.375	0.036	0.037	<0.05
Thiol μmol/L	1204.58 ± 173.62	1093.93 ± 73.34	756.10 ± 64.27	691.5	14.1	16.45	<0.05
Selenium μg/L	74.45 ± 18.41	72.77 ± 18.57	41.54 ± 8.33	10.5	1.31	1.3	<0.05

Results are expressed as mean ± SE of the mean.

Methods used to detect apoptosis

1. Cellular morphology by light microscope, phase contrast microscope, and interference contrast microscope (11,12).

2. Nuclear morphology and DNA quantitation after feulgen cytochemical reaction (13,14).

3. Biochemical changes associated with lymphocytes apoptosis:

a. Malondialdehyde (15), final lipid peroxidation product.

b. Thiol protein (16).

c. Selenium measured by atomic absorption spectrophotometry.

RESULTS

The results are expressed under two distinct lines:

I. Cellular changes include viability, morphology and DNA quantitation.

II. Biochemical changes include lipid peroxidation activity assessed by MDA, and scavenger system assessed by thiol protein and selenium level.

Control group

In this group the non-viable lymphocyte obtained from 100 cells for each individual subject of the normal was 4.22±0.51, 40.72±7.11, 100±0.0, at time zero, after 24 and 72 hours incubation respectively. The cellular morphology was assessed by phase contrast microscopy, the apoptotic processes were followed as such stage I: membrane bleb formation, stage II: flattening of cells with protrusion of echinoid spikes and stage III: formation of membrane blisters and disintegration of cells into apoptotic bodies (11,17). The progress in apoptotic process was time dependent as what has been observed by Willingham (11) and Collins *et al.* (17) as shown in Table 1.

Incubation of lymphocytes with H_2O_2, after 10 minutes (11.66±2.40 vs. 3.36±0.54) lymphocytes were apoptotic, after 24 hours all lymphocytes were apoptotic the difference was statistically significant. There was a rapid reduction in DNA contents (140.27±8.35 O.D unit x 10^{-3}), obtained after only 24 hours.

These changes are associated with significant increase in MDA production, depletion of thiol and selenium (Table 2).

Incubation of lymphocytes with antioxidants

Cell viability: After 72 hours incubation (57.27 ± 10.68 and 69.87 ± 10.21) of lymphocytes were apoptotic with Zn and Cu respectively vs. 99.66 cells died without them, which was significant statistically at $p < 0.05$.

The DNA contents were significantly preserved, it was (158.71 ± 14.61, 150.24 ± 11.3 O.D unit x 10^{-3}) after 72 hours incubation with Zn and Cu respectively vs. (140.62 O.D unit x 10^{-3}) without them.

MDA level was (3.34 ± 42, 3.43 ± 0.4 nmol/ml) after 72 hours incubation with Zn and Cu respectively vs. (5.27 nmol/ml) without them.

Thiol proteins were significantly preserved which were (920.7 ± 80.72, 886.48 ± 70.43 μmol/L) after 72 hours incubation with Zn and Cu respectively vs. (747.45 μmol/L) without them.

Selenium was reduced to (52.4 ± 3.2 and 66.17 ± 2.4 μg/L) after 72 hours incubation with Zn and Cu respectively vs. (42.25 μg/L) without these metals which show significant statistical difference (Table 3).

Table 3: Effects of zinc and copper on the cellular and biochemical changes related to lymphocyte apoptosis.

N=31		0 h	24 h	72 h	p value	B1	B2	Bt
TBE	Zn	3.36±0.54	28.93±12.96	57.27±10.68	<0.05	1.06	0.59	0.726
	Cu	3.36±0.54	33.39±14.87	69.87±10.21	<0.05	1.25	0.76	0.936
DNA	Zn	207.98±18.96	161.14±13.46	158.71±14.61	<0.05	1.94	0.056	0.594
	Cu	207.98±18.96	163.61±18.7	150.24±11.3	<0.05	1.84	0.27	0.727
MDA (nmol/mL)	Zn	2.96±0.8	2.93±0.4	3.34±0.4	<0.05	0.0013	0.0094	0.0067
	Cu	2.96±0.8	2.97±0.4	3.43±0.42	<0.05	0.0006	0.0094	0.0069
Thiol (μmol/L)	Zn	1204.58±173.62	1043.51±78.71	920.7±80.72	<0.05	6.71	2.55	3.744
	Cu	1204.58±173.62	1006.93±77.64	886.48±70.43	<0.05	8.23	2.5	4.14
Se (μg/L)	Zn	74.45±18.4	70.31±4.1	52.4±3.2	<0.05	0.172	0.373	0.315
	Cu	74.45±18.4	69.41±1.3	66.17±2.4	<0.05	0.21	0.067	0.108

Results are expressed as mean ± SE of the mean.
B1 = Rate of changes at 1st incubation period (0 - 24h).
B2 = Rate of changes at 2nd incubation period (24 - 72h).
Bt = Rate of changes at both incubations period (0 - 72h).

DISCUSSION

In this study apoptosis was associated with generation of free radicals since there was increased MDA production, consumption of thiol and selenium (10). Generation of free radicals with apoptotic process was suggested by Hildeman *et al.* (18).

PCD was induced by addition of H_2O_2, which has direct damaging effect on membrane lipids leading to membrane lipid peroxidation (2). H_2O_2 can enter the cell to lead to release cytochrome c, which can directly stimulate caspase 3 (2). Together with cytochrome c they execute apoptotic process (4).

Inhibition of apoptotic process by antioxidants

Effect of zinc : Zn inactivates caspase 3, either directly or indirectly by inhibiting caspase 6 which activates procaspase 3 (5).

Zn can increase BCl_2/Bax ratio, thus inhibits the release of cytochrome c from mitochondria (19). Zn can inhibit Ca/Mg dependent endonuclease which cleave DNA (20, 21). Leccia *et al.* (22) found that the addition of $ZnCl_2$ to the culture media significantly reduces the rate of apoptosis.

Zn can stabilize SH-group of protein (6) and acts as a free radical scavenger as part of the enzyme Cu/Zn SOD (21). Zn can antagonize redox-active transition metals such as iron (1).

Effect of copper : The action of copper through the enzyme Cu/Zn SOD is to act as a scavenger for superoxide anion (2). Ceruloplasmin acts as a scavenger against oxidative stress, this action is independent of its action as Cu carrier (8).

A further study is suggested to study the effect of Zn status *in vivo* and its effect on the *in vitro* culture. Further study suggested on Zn and Cu is to know the precise pathway by which they inhibit apoptotic process.

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