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## Increased Susceptibility to Oxidative Death of Lymphocytes from Alzheimer Patients Correlates with Dementia Severity

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### Abstract

We previously reported on enhanced susceptibility to death of lymphocytes from Alzheimer's disease (AD) patients when exposed to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)-induced oxidative stress and an increased resistance to death in those of patients with a history of skin cancer. This is consistent with our hypothesis proposing that the cellular machinery controlling cell death is deregulated in opposite directions in Alzheimer's disease (AD) and cancer, to explain the inverse association observed in epidemiological studies. Here we investigated whether the observed increased susceptibility correlates with the degree of dementia severity. Peripheral lymphocytes from 23 AD patients, classified using the Clinical Dementia Rating (CDR) into severe dementia (CDR 3, n=10)

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### CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

### DISCLOSURE STATEMENT

None of the authors have conflicts of interest related to this study.

### AUTHORS' CONTRIBUTIONS

Daniela P Ponce: Performed research, designed experiments, collected data, analyzed data, revised paper.

Felipe Salech: Performed research, designed experiments, collected data, analyzed data, revised paper.

Mónica Silva: Performed research, collected data.

Chengjie Xiong: Analyzed data, revised paper.

Catherine M Roe: Analyzed data, revised paper.

Mauricio Henriquez: Designed experiments, analyzed data, revised paper.

Andrew FG Quest: Designed experiments, analyzed data, contributed important reagents, and revised paper.

María Isabel Behrens: Directed the project, designed experiments, contributed important reagents, analyzed data, wrote paper.

and mild-to-moderate dementia (CDR 1–2, n=13), and 15 healthy controls (HC) (CDR 0), were exposed to H<sub>2</sub>O<sub>2</sub> for 20 hours. Lymphocyte death was determined by flow cytometry and propidium iodide staining. The greatest susceptibility to H<sub>2</sub>O<sub>2</sub>-induced death was observed for lymphocytes from severe dementia patients, whereas those with mild-to-moderate dementia exhibited intermediate values, compared to healthy controls. A significant increase in the apoptosis/necrosis ratio was found in AD patients. Poly (ADP-ribose) polymerase-1 (PARP-1) inhibition significantly protected from H<sub>2</sub>O<sub>2</sub>-induced death of lymphocytes, whereby a lower degree of protection was observed in severe AD patients. Moreover, inhibition of PARP-1 abolished the differences in apoptosis/necrosis ratios observed between the three groups of patients. These results support the notion that AD is a systemic disorder, whereby enhanced susceptibility to H<sub>2</sub>O<sub>2</sub>-induced death in peripheral lymphocytes correlates with dementia severity and enhanced death in AD patients is attributable to a PARP-dependent increase in the apoptosis/necrosis ratio.

### Keywords

Alzheimer; apoptosis; dementia; necrosis; PARP-dependent cell death; peripheral lymphocytes

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## INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the most frequent cause of dementia. The causes of the disease are unknown but many factors are implicated, including toxic effects of amyloid and tau [1, 2], inflammation [3] and oxidative damage [4]. Cellular aging and neurodegenerative diseases lead to increased generation of reactive oxygen species (ROS) that modify biomolecules, diminishing the normal functions of neuronal cells and eventually leading to neuronal loss in AD. In epidemiological studies we and others have found that AD and cancer are inversely associated, such that patients with a history of cancer in the past have a reduced risk of developing Alzheimer type dementia and inversely, those with AD have a reduced risk of developing cancer in the future [5–12]. In the search for molecular mechanisms to explain the inverse epidemiological relationship observed between Alzheimer Disease (AD) and cancer we have proposed that the cellular machinery controlling cell death might be deregulated in opposite directions to explain the mutual protection observed between the two disorders. Changes in the expression of key molecules involved in the regulation of cell cycle or cell death/survival, such as p53, Pin1, wnt [12–14] may explain these observations. We previously reported that lymphocytes from AD patients are more susceptible to oxidative cell death induced by H<sub>2</sub>O<sub>2</sub> exposure than those from control subjects [15]. Using flow cytometry, electron microscopy and by measuring caspase activity, we determined that H<sub>2</sub>O<sub>2</sub> exposition induced both necrotic and apoptotic death of lymphocytes, the latter being independent of caspase activity and dependent on poly (ADP-ribose) polymerase-1 (PARP-1) activity [16]. To understand the relationship between lymphocyte death and normal aging, we evaluated the susceptibility to H<sub>2</sub>O<sub>2</sub>-induced death of lymphocytes from healthy subjects of ages ranging from 24–95 years [16]. We showed that aging was not associated with an overall increase in lymphocyte susceptibility to death, but that there was a change in the pattern of cell death: necrotic death prevailed in healthy young subjects, while in cognitively normal aged subjects there was an

increase in apoptotic death. As a consequence, an increase in the apoptosis/necrosis ratio was observed with age [16]. Moreover, we determined that PARP-1 inhibition with 3-Aminobenzamide (3-ABA) provided significant protection against H<sub>2</sub>O<sub>2</sub>-induced cell death, whereby protection tended to be lower in patients with AD [15, 16]. In this study we evaluated whether the severity of dementia in AD patients correlates with the susceptibility to H<sub>2</sub>O<sub>2</sub>-induced cell death and changes in the apoptosis/necrosis ratio. In addition, we explored whether the protection provoked by PARP-1 inhibition varies with the degree of dementia severity.

## MATERIALS

RPMI 1640 medium and fetal bovine serum were from Biological Industries (Kibbutz Beit-Haemek, Israel). Ficollpaque™ PREMIUM was from GE Healthcare (Little Chalfont, UK). The H<sub>2</sub>O<sub>2</sub> was from Merck (Darmstadt, Germany). N-acetyl cysteine (NAC), sodium 4,5-dihydroxybenzene-1,3-disulfonate (Tiron) and 3-Aminobenzamide (3-ABA) were from Sigma Chemical (St Louis, MO). The antioxidant EUK-134 and TBARS assay kit were from Cayman chemical (Ann Arbor, Mi). OxiSelect™ Glutathione Reductase Assay Kit was from Cell Biolab (San Diego, CA). Fluorescence-activated cell sorting (FACS) was from Becton Dickinson (Mountain View, CA).

## PATIENTS

A total of 38 individuals, 23 AD patients –10 severe dementia (CDR 3), 13 mild-to-moderate dementia (CDR 1–2), and 15 healthy donors (CDR 0) were recruited after providing informed consent approved by the Ethics Committee of the Hospital Clínico de la Universidad de Chile. In severe cases of dementia, the consent was provided by their caregivers. Care was taken to avoid performing the H<sub>2</sub>O<sub>2</sub> experiments in lymphocytes from patients with signs of infection or inflammation, because this might alter lymphocyte susceptibility. Five patients from the control group, 3 from the mild-moderate group and 1 from the severe dementia group were also analyzed in the previous manuscript (Behrens et al 2012). However they donated blood samples for new experiments in this study. AD diagnosis was established following the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria [17] and dementia severity was rated with the CDR, whereby CDR 1, 2 and 3 indicates mild, moderate, and severe dementia, respectively, and CDR 0 corresponds to the absence of any cognitive impairment [18], the Mini-mental State Examination (MMSE) [19] and the Montreal Cognitive Assessment (MoCA) [20]. Maximum score for MMSE and MoCA is 30, with lower scores associated with greater cognitive deterioration (Table 1). Healthy controls were submitted to the same neurological and neuropsychological evaluations.

## METHODS

Peripheral blood lymphocytes were obtained by venopuncture (15 ml) and extracted by Ficoll-Hypaque density centrifugation [16] and exposed to increasing concentrations of H<sub>2</sub>O<sub>2</sub> for 20 hours, in the presence or absence of the PARP-1 inhibitor 3-Aminobenzamide

(3-ABA) 5mM. The samples were normalized to cell count at a density of  $1 \times 10^6$  cells per ml. In a different set of experiments the protection of lymphocytes from ROS-mediated damage conferred by PARP-1 inhibition using 3-ABA was evaluated in parallel with treatments using different antioxidants applied 1 hour before  $H_2O_2$  treatment; N-acetyl cysteine (NAC), 5mM, EUK-134, 20  $\mu$ M, and Sodium 4,5-dihydroxybenzene-1,3-disulfonate (Tiron) 4mM (3–5 patients per group). Cell death was evaluated by flow cytometry following propidium iodide (PI) staining, whereby viable (PI-negative), apoptotic (PI-positive, hypodiploid) and necrotic (PI-positive diploid) cells were distinguished [16]. Apoptotic death was corroborated with concomitant annexin V staining [21]. Cellular damage induced by oxidative stress was determined using thiobarbituric acid reactive substances (TBARs) assay in freshly extracted lymphocytes from AD and healthy donors following instructions provided by the manufacturer. Glutathione reductase activity was measured with the assay kit OxiSelect™. Reduced glutathione (GSH) and oxidized glutathione (GSSG) levels in lymphocytes were assayed by fluorimetry as previously described [17]. The results were expressed in nmol of GSH and GSSG per milligram of protein.

### Statistical Analysis

Differences between the three experimental groups at each dose in lymphocyte survival, apoptosis, and necrosis were analyzed using a SPSS general linear model with Bonferroni correction, and the data were adjusted for age and sex. Results were expressed as means  $\pm$  standard error of the mean (SEM). Differences  $p < 0.05$  were considered statistically significant.

## RESULTS

A significant interaction between  $H_2O_2$  concentrations and CDR groups was found for lymphocyte survival ( $p < 0.005$ ). Pairwise comparisons indicated that upon exposure to  $H_2O_2$ , lymphocytes from AD patients showed increased susceptibility to death compared with control lymphocytes. The dose-response curves of lymphocyte viability, at concentrations of  $H_2O_2$  ranging from 10  $\mu$ M to 30 mM, were shifted to the left (enhanced sensitivity) for lymphocytes from AD patients compared to HC (Fig. 1A). A larger shift was observed for lymphocytes from severe AD patients and intermediate for lymphocytes from mild-to-moderate AD patients. Upon treatment with 100  $\mu$ M  $H_2O_2$ , survival values were  $20 \pm 4\%$ ,  $33 \pm 3\%$  and  $47 \pm 3\%$  for severe dementia, mild-to-moderate dementia, and HC lymphocytes, respectively (mean  $\pm$  SEM, Fig. 1Ainset). Lower MMSE scores were associated with lower lymphocyte survival values upon exposure to 100  $\mu$ M  $H_2O_2$ , with a significant correlation (2-tailed) with Kendall's tau-b (0.389;  $p < 0.001$ ) and Spearman-Rho correlation (0.566,  $p < 0.0001$ ) (Fig. 1B). A similar correlation was obtained for the MoCA, Kendall's tau-b 2-tailed (0.449;  $p < 0.0001$ ) and Spearman-Rho 2-tailed test (0.622,  $p < 0.0001$ ) (supplementary material Fig. 1). Treating MMSE as a categorical variable, using Chilean published cut-offs [22] also rendered significant differences between healthy controls and mild/moderate and severe AD (but not between mild/moderate and severe AD, data not shown).

The increased cell death in AD patients was due to higher levels of apoptosis, corroborated by PI and annexin co-staining (Fig. 2A and B). The ratios of apoptotic to necrotic (A/N) death of lymphocytes calculated for each H<sub>2</sub>O<sub>2</sub> concentration showed that A/N ratios were significantly greater in AD patients than in HC (Fig. 2C). Since we had previously reported that aging is associated with an increase in the A/N ratios of lymphocyte death in HC, we evaluated whether age affected the A/N ratio in AD patients. Results shown in Fig. 2D show that no apparent relationship exists between age and the A/N ratio measured at 100 μM H<sub>2</sub>O<sub>2</sub>, neither in AD nor HC lymphocytes (Fig. 2D).

Because we have previously observed a potent protection of H<sub>2</sub>O<sub>2</sub>-induced death of lymphocytes with PARP-1 inhibition with 3-ABA, we tested the effects of this inhibitor in lymphocytes from patients with different dementia severity. PARP-1 inhibition with 3-ABA significantly protected lymphocytes from H<sub>2</sub>O<sub>2</sub>-induced cell death, and the extent of protection was significantly lower for lymphocytes from severe dementia patients (Fig. 3A). Furthermore, upon PARP-1 inhibition, the A/N ratios were equivalent to 1 in the three groups of patients tested at different H<sub>2</sub>O<sub>2</sub> concentrations (Fig. 3B). To further explore the mechanism of H<sub>2</sub>O<sub>2</sub>-induced damage, in a different set of experiments, we tested the effect of 3-ABA in parallel with the effect of antioxidants known to act at different cellular sites; N-acetyl cysteine (NAC), a precursor in the formation of the antioxidant glutathione, EUK-134, a superoxide dismutase mimetic, and Sodium 4,5-dihydroxybenzene-1,3-disulfonate (Tiron), a free radical scavenger. The three antioxidants provided approximately 80% protection against H<sub>2</sub>O<sub>2</sub>-induced death at 100 μM concentration, but were not protective at higher H<sub>2</sub>O<sub>2</sub> concentrations (Fig. 2 supplementary material). Instead 3-ABA conferred more than 80% protection up to 1 mM H<sub>2</sub>O<sub>2</sub> concentration, suggesting that these effects occurred downstream of antioxidant action (Fig. 2 supplementary material and Fig. 3A).

In accordance with data in the literature [23], levels of lipid peroxidation as a marker of cellular oxidative damage were elevated in lymphocytes from AD patients compared to controls (Supplementary Fig. 3). The intrinsic antioxidant machinery of lymphocytes from AD patients was measured as activity of glutathione reductase (GRD) and GSH/GSSG ratios. Neither GRD activity nor the GSH/GSSG ratios were significantly different in lymphocytes from AD patients compared to controls (Supplementary Fig. 3).

## DISCUSSION

Overall the data presented here show that increased susceptibility to apoptotic death of lymphocytes from AD patients after oxidative damage by exposure to H<sub>2</sub>O<sub>2</sub> correlates with the degree of dementia severity. The augmented susceptibility was observed both as an increase in the overall percentage of death and as an increase in the A/N ratios. The increase in cell death correlated well with the cognitive impairment independent of the method of

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### SUPPLEMENTARY MATERIALS

Supplementary Fig. (1). Lymphocyte survival under oxidative stress plotted against cognitive status measured with MoCA test.

Supplementary Fig. (2). Effect of antioxidants on H<sub>2</sub>O<sub>2</sub>-induced death of lymphocytes from AD patients.

Supplementary Fig. (3). Oxidative stress and antioxidant defenses in lymphocytes from AD patients.

Supplementary material is available on the publisher's web site along with the published article.

quantification used (CDR and MMSE or MoCA). These data reinforce conclusions from our previous studies which identified a higher susceptibility of lymphocytes from AD patients to cell death induced by H<sub>2</sub>O<sub>2</sub> [15].

The increase in A/N ratios observed in AD lymphocytes is not due to differences in the age of patients with severe AD, and was greater than expected to occur by aging [16]. In our previous report we observed an increase in apoptosis/ necrosis ratio associated with age, when measured at ages ranging from 24 to 95 years; the smaller differences in age of the patients in this study (from 63 to 89 years) did not permit observing age-dependent variations.

These results also support the notion that AD is a systemic disorder [24], in which alterations in susceptibility to cell death are also observed for cells that are not part of the nervous system. Our results support the idea that lymphocytes isolated from the blood provide peripheral correlates of brain function [25, 26]. It is important to mention that these results do not allow us to distinguish whether increased susceptibility to cell death is a reflection of the progression of the brain disorder, or whether this change plays a causative role in the genesis of the disease state.

Several studies in the literature have reported on increased lipid peroxidation and decreased antioxidant defenses in plasma and blood cells of AD patients compared to controls [23, 27–29]. Accordingly, we found increased lipid peroxidation in lymphocytes from AD patients. Decreased activity of GRD has been reported in erythrocytes and in immortalized lymphocytes of AD patients [28, 30] and some reports have shown reduced GSH/GSSG ratios in AD lymphocytes [31] but not others [32]. We did not observe significant differences in GRD activity nor GSH/GSSG ratios in AD lymphocytes compared to controls.

Other reports in the literature have observed increased death of peripheral lymphocytes of AD patients [33–36], and defects in G1/S cell cycle checkpoint regulation [37, 38]. The increase in apoptosis may reflect a change in the activity or expression of key molecules belonging to the cellular machinery of protection against oxidative damage, such as molecules involved in cell cycle or cell survival/death mechanisms; p53, PARP, PIN1, and wnt [12–14]. For example, increased levels of p53 have been reported in peripheral lymphocytes of AD patients [39]. A recent report found several genes and pathways, among them p53, wnt signaling and Pin1, that were deregulated in opposite directions in cancer and AD [40]. It is intriguing to speculate that the increases in apoptotic death of lymphocytes detected as the disease progresses in AD patients may be due to increased p53 levels in more advanced stages of dementia. In this perspective, it is interesting to mention a recent report demonstrating that p53 is involved not only in triggering apoptosis, but also in the regulation of necrotic, PARP-dependent cell death [39, 41].

PARP-1 is a nuclear enzyme that polymerizes multiple adenosine diphosphate (ADP)-ribose molecules to nuclear proteins consuming large amounts of nicotinamide adenine dinucleotide (NAD<sup>+</sup>) that are also essential for processes like DNA repair. Under conditions where severe DNA damage is inflicted, such as under conditions of exacerbated oxidative



stress, PARP-1 activation becomes excessive, depleting the cell of NAD<sup>+</sup> and ATP, and promoting a form of caspase-independent cell death, coined parthanatos [42]. In our previous studies we observed that PARP inhibition protected the cells from H<sub>2</sub>O<sub>2</sub>-induced cell death, whereby a tendency (not statistically significant) towards a lower degree of protection was detectable in AD lymphocytes [15]. In that study, results were obtained using samples from AD patients with mild, moderate and severe disease that were considered as a single group. In the studies presented here, we found that by separating dementia cases according to severity, significant differences became detectable and that lymphocytes obtained from the severely demented group of AD patients were significantly more susceptible to oxidative stress induced damage (and death), further strengthening the working hypothesis that lymphocytes from AD patients are more susceptible to this atypical PARP-dependent type of death. The presence of antioxidants such as Tiron, EUK-134 and NAC protected against H<sub>2</sub>O<sub>2</sub>-induced death at concentrations of 100 μM, but not at higher H<sub>2</sub>O<sub>2</sub> concentrations. This suggests that PARP-1 action, at the DNA level, is downstream of the effect of the antioxidants. Excessive PARP-1 activation has been linked to the progression of several chronic diseases and neurodegenerative disorders [42, 43], and more recently PARP was implicated in AD pathogenesis [44, 45]. Inhibition of PARP has been extensively explored in the perspective of developing effective treatments for cancer [46], and our observations suggest this target may deserve further attention in the treatment of Alzheimer's disease.

Finally, Alzheimer's disease (AD), the most frequent cause of dementia, is characterized by the lack of adequate biomarkers for disease diagnosis and objective stratification of disease severity. Bearing in mind our results, we propose that measurement of lymphocyte death induced by H<sub>2</sub>O<sub>2</sub> may be exploited as a biological marker of AD severity, although further research is needed to corroborate this potential.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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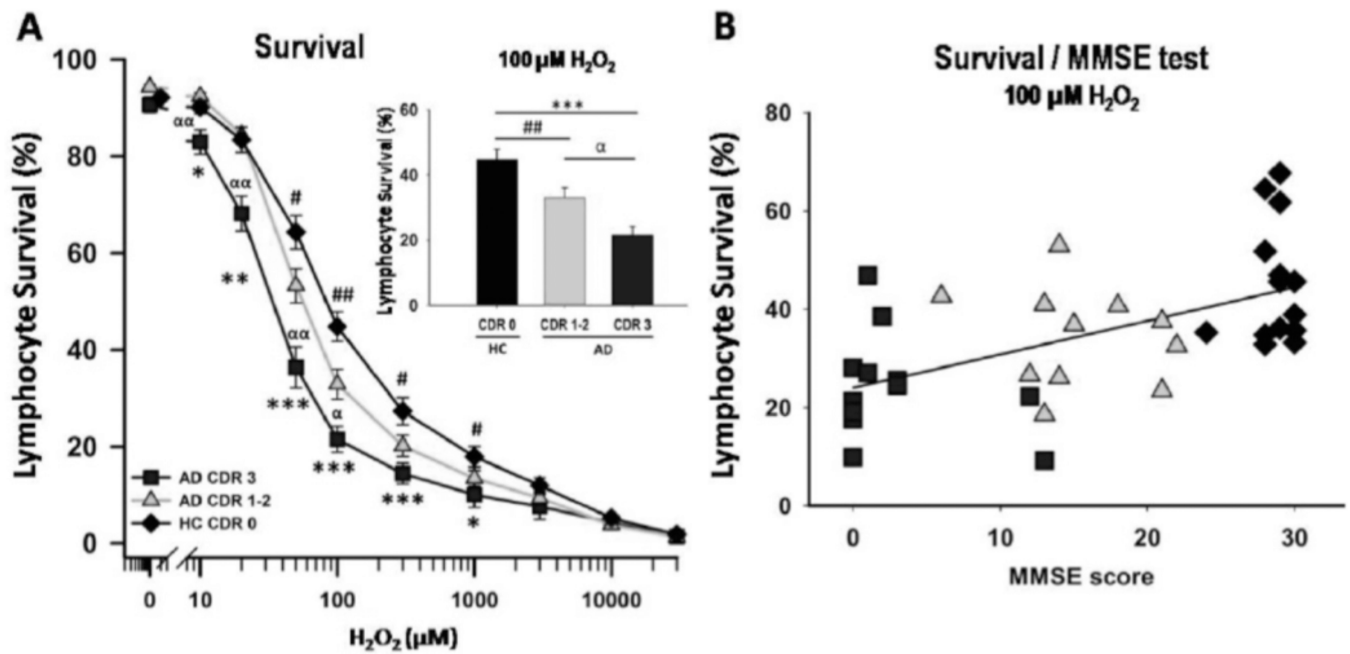
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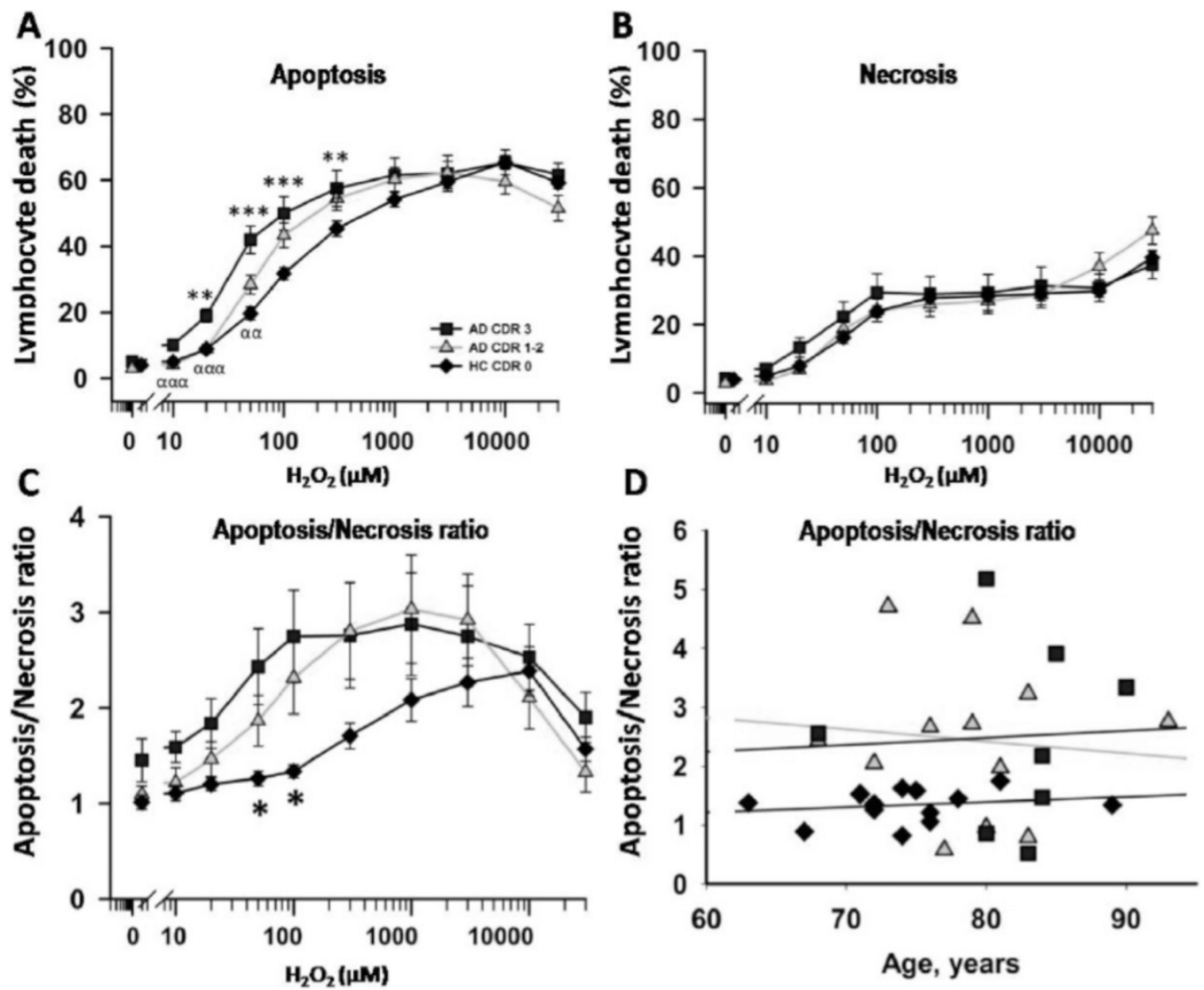
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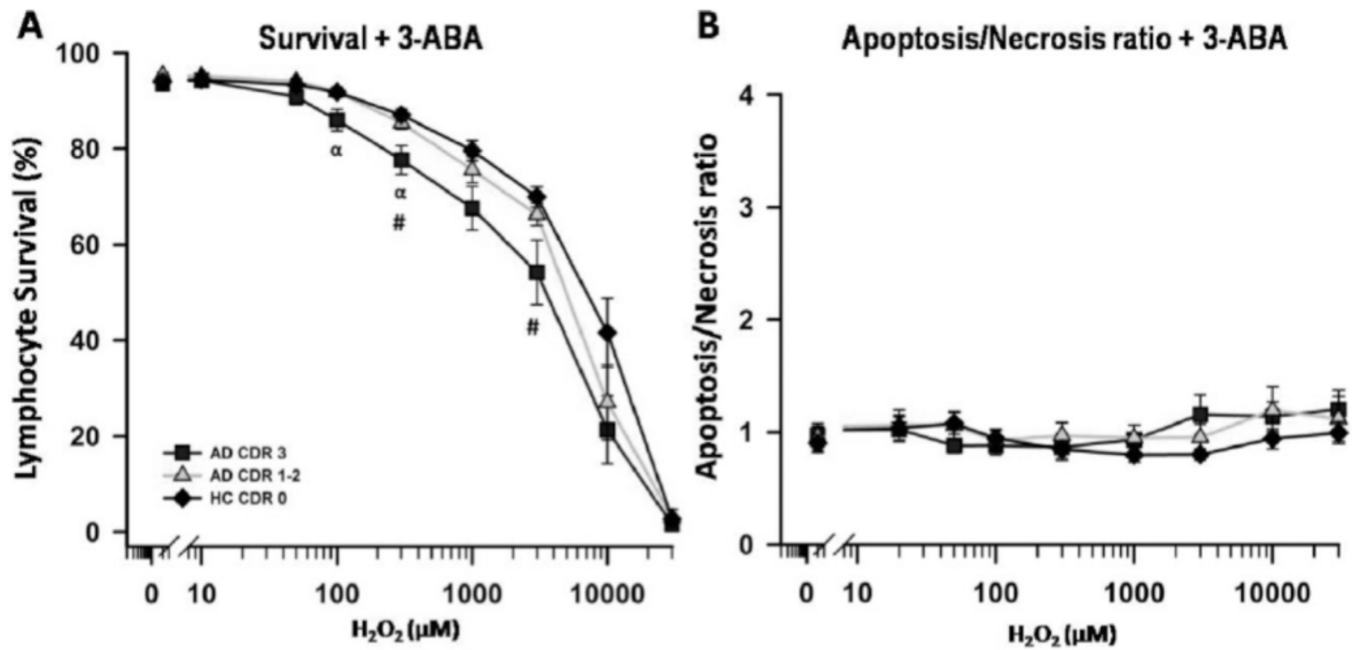


**Fig. (1).**

H<sub>2</sub>O<sub>2</sub>-induced death of peripheral lymphocytes from AD patients correlated with the degree of dementia severity. Lymphocytes from 10 severe dementia AD patients (CDR 3) (squares), 13 mild-to-moderate AD (CDR 1–2) (triangles), and 15 healthy controls (rhomboids) were exposed to H<sub>2</sub>O<sub>2</sub> for 20 h and cell death was determined by flow cytometry and propidium iodide staining. **A:** Survival values. Means ± S.E. \* =AD CDR 3 vs HC; # =AD CDR 1–2 vs HC; α =AD CDR 3 vs AD CDR 1–2. One symbol: p<0.05; 2 symbols: p<0.005; 3 symbols: p<0.0001. Inset: Lymphocyte survival values (% mean ± SEM) measured at 100 μM H<sub>2</sub>O<sub>2</sub>, in the three groups of patients. **B:** Survival values measured at 100 μM H<sub>2</sub>O<sub>2</sub> in lymphocytes from individual participants plotted against their MMSE scores. Kendall's tau-b 2-tailed correlation (0.389; p<0.001). Symbols as in (A).



**Fig. (2).** Apoptotic/necrotic ratios after H<sub>2</sub>O<sub>2</sub>-induced death of peripheral lymphocytes. **A and B:** Apoptosis and necrosis values measured in the experiments in Fig. (1A). **C:** Apoptosis/necrosis ratios calculated at each H<sub>2</sub>O<sub>2</sub> concentrations in the three groups of patients (mean ± SEM). **D:** Apoptosis/necrosis ratios of individual participants in the three groups plotted against age. Symbols as in Fig. (1A).



**Fig. (3).** H<sub>2</sub>O<sub>2</sub>-induced death of peripheral lymphocytes in the presence of 3-ABA, a PARP-1 inhibitor. PARP-1 inhibition induced a marked protection from H<sub>2</sub>O<sub>2</sub>-induced death, with a significantly reduced degree of protection in severe AD lymphocytes (**A**). **B**: Apoptosis/necrosis ratios measured in the presence of PARP-1 inhibition in the three groups of patients. Symbols as in Fig. (1A).

**Table 1**

Demographic data of study participants.

	Healthy Controls CDR 0	AD CDR 1-2	AD CDR 3
	N=15	N=13	N=10
Age, y mean $\pm$ SE	75.7 $\pm$ 1.7	77.1 $\pm$ 2.3	80.7 $\pm$ 2.3
Age range, y	63-89	68-93	68-90
Female sex, (%)	12 (80)	10 (77)	10 (100)
Education, y	10.9 $\pm$ 1.7	10.0 $\pm$ 1.2	9.9 $\pm$ 0.9
MoCA <sup>1</sup> test	26.1 $\pm$ 0.7	10.3 $\pm$ 1.8	1.4 $\pm$ 0.8
MMSE <sup>2</sup>	29.0 $\pm$ 0.3	15.7 $\pm$ 1.4	3.9 $\pm$ 1.9
Hypertension (%)	4 (27)	10 (69)	6 (60)
Diabetes (%)	3 (20)	2 (13)	5 (50)
Tobacco	4 (27)	4 (27)	4 (40)
Cholinesterase inhibitors (%)	NA	9 (69)	4 (40)
Memantine (%)	NA	8 (61)	3 (30)

<sup>1</sup>Montreal Cognitive Assessment.<sup>2</sup>Mini-mental State Examination.

NA = not applicable.