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## Accepted Manuscript

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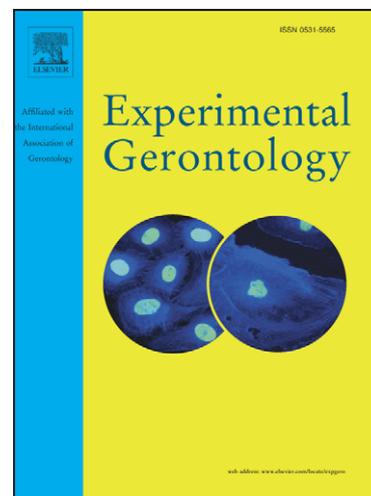
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## Effect of zinc on cellular poly(ADP-ribosyl)ation capacity

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**Key words**

PARP-1, Poly(ADP-ribosyl)ation capacity, zinc supplementation, aging

**Abstract**

Poly(ADP-ribosyl)ation is a posttranslational protein modification, which is catalyzed by poly(ADP-ribose) polymerase-1 (PARP-1) and plays a role in DNA repair and maintenance of genomic stability. A decrease in cellular poly(ADP-ribosyl)ation has been implicated in the aging process. As PARP-1 is a zinc finger protein its decreased function might be related to age-related zinc deficiency. To test this hypothesis we assessed cellular poly(ADP-ribosyl)ation capacity in 29 donors from Greece, Italy and Poland as function of age and nutritional zinc status. Our results reveal a positive correlation between cellular poly(ADP-ribosyl)ation capacity and zinc status in human peripheral blood mononuclear cells (PBMC) ( $p < 0.05$ ). We could also confirm a decrease of PARP-1 activity with donor age, highlighting the role of PARP in the aging process. The results demonstrate that zinc supplementation in elderly people can increase the cellular poly(ADP-ribosyl)ation capacity of their PBMC. We speculate that this may help maintain integrity and stability of the genome more efficiently and so contribute to an extension of healthspan.

## Introduction

Poly(ADP-ribosyl)ation is a reversible posttranslational modification of nuclear proteins occurring as an early cellular response to DNA damage generated by endogenous and exogenous damaging agents in mammalian cells (Lindahl et al., 1995). The family of poly(ADP-ribose) polymerases (PARPs) catalyze the synthesis of ADP-ribose units, using NAD<sup>+</sup> as substrate and attach them covalently to “acceptor” proteins like DNA repair enzymes, histones and, in the case of PARP-1, mostly the enzyme itself (Adamietz and Rudolph, 1984; Ogata et al., 1981). Poly(ADP-ribosyl)ation is involved in several cellular processes including DNA repair and maintenance of genomic stability. An involvement of poly(ADP-ribose) metabolism in the aging process has long been suggested, based on the observation that the cellular capacity to produce poly(ADP-ribose) in peripheral blood mononuclear cells (PBMC) correlates positively with species-specific life span in mammals (Grube and Burkle, 1992). Furthermore we were able to establish an association between high cellular poly(ADP-ribosyl)ation capacity in lymphoblastoid cells with human longevity (Muiras et al., 1998). On the other hand, cellular poly(ADP-ribosyl)ation capacity decreased with age in rats (Grube and Burkle, 1992) and in humans (Chevanne et al., 2007; Grube and Burkle, 1992). Poly(ADP-ribosyl)ation is mostly catalyzed by PARP-1, an abundant nuclear enzyme that binds via its zinc finger motifs to DNA with single or double strand breaks. Zinc binding has shown to be essential for PARP-1 activation (Mazen et al., 1989).

The distribution of zinc ions may have an impact on processes related with DNA repair and maintenance of genomic integrity and stability, and thus in the aging process (Vasto et al., 2006). It is quite obvious that free zinc influences the activation of zinc finger proteins and that during aging the intake of zinc decreases (Mocchegiani et al., 2006). These facts underpin the hypothesis that there is a link between cellular poly(ADP-ribosyl)ation capacity and zinc content in cells and that zinc could be a limiting factor for the enzymatic activity of PARP-1.

To address this question, we measured cellular poly(ADP-ribosyl)ation in PBMC from elderly donors as function of plasma zinc concentrations before and after a 7-week course of oral zinc supplementation.

## Materials and methods

### Subjects:

Twenty-nine healthy old subjects from three different European countries (Greece, Italy and Poland) were recruited in the framework of the ZINCAGE project (supported by the EU Commission) and supplemented with 10 mg zinc aspartate (Unizink from Köhler, Alsbach, Germany; or ZINCAS from FARMAPOL, Poland) for  $48 \pm 2$  days. Blood was taken before and after zinc supplementation and the cellular zinc level was determined (Cipriano et al., 2006). We assessed cellular poly(ADP-ribosyl)ation capacity by a recently established flow cytometry based assay (Kunzmann et al., 2006) in 29 different donors recruited in Italy, Poland and Greece as function of donor age and nutritional zinc status.

### Separation of peripheral blood mononuclear cells:

Peripheral blood (about 15 ml) was centrifuged at  $450 \times g$  for 10 min at  $4^\circ\text{C}$  to separate plasma. The plasma was collected into 1-2 ml vials and stored at  $-80^\circ\text{C}$ . The remaining blood was diluted 1:3 with phosphate buffered saline (1 x PBS), pH 7.4, without  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  (Dulbecco A; Oxoid, Basingstoke, UK), and was carefully stratified with a pipette on top of a Ficoll-Hypaque solution ( $d = 1.077$  g/ml) (Biochrome AG, Berlin, Germany) at room temperature, in a 15 ml centrifuge tube. The solution was centrifuged at  $450 \times g$  for 30 min at  $20^\circ\text{C}$ . The mononuclear cell layer was recovered and washed two times with PBS (by two centrifugation steps at  $450 \times g$  for 10 min).

**Cryopreservation:**

PBMC were counted, 5 ml of PBS was added and cells were centrifuged at 450 x *g* for 10 min; then the tube was left on ice for 10-20 min. The supernatant was removed and cells were resuspended in Fetal Calf Serum (FCS) (Invitrogen-Gibco, San Giuliano, Italy) containing 5% dimethyl sulfoxide (DMSO) (MP-Biomedicals, Eschwege, Germany). After 5 min further DMSO was added to a final concentration of 10%. Then PBMC were immediately placed in a freezing container with isopropanol and put in a  $-80^{\circ}\text{C}$  freezer over night. Finally the cells were placed in liquid nitrogen.

**Cryopreserved PBMC recovery:**

PBMC were recovered by submersion in a  $37^{\circ}\text{C}$  water bath until the ice was melted. The cells were transferred to prechilled phosphate buffered saline (PBS) and were centrifuged at 228 x *g* for 10 min at  $0^{\circ}\text{C}$ . The cells were resuspended in cold PBS and checked for cell number by CASY (Schärfe System, Germany).

**Determination of zinc:**

All plasma samples and standard were diluted 1:10 with a diluent containing the following reagents: 0.1% Triton and 0.15 %  $\text{HNO}_3$  (Sigma Aldrich, Buchs, Switzerland). External calibration solutions (VWR Italia, Milano, Italy)

containing Zn (blank to 2,000 ppb) were prepared by serial dilution of a parent 1,000 ppm stock, using the same diluent used to dilute the samples.

Measurement of plasma zinc was performed with a Thermo XII Series ICP-MS (Thermo Electron Corporation, Waltham, MA, USA). The instrument was operated with a Peltier cooled impact bead spray chamber, single piece quartz torch (1.5 mm i.d. injector) together with Xi interface cones and a Cetac-ASX 100 autosampler (CETAC Technologies, Omaha, Nebraska, USA). A Burgener Trace nebulizer was used as this device does not block during aspiration of clinical samples. The instrument was operated in standard mode (non-CCT) which is the preferred method for acquiring data for Zn66. The instrument was operated using 1,400 W RF power, 1.10 L min<sup>-1</sup> nebuliser gas flow, 0.70 L min<sup>-1</sup> auxiliary gas flow, 13.0 L min<sup>-1</sup> cool gas flow, 70 ms dwell time, 30 s sample uptake and 35 s wash time (2 repeats per sample).

#### **PARP activity assay:**

This assay was performed exactly as described previously (Kunzmann et al., 2006). Briefly, cells were permeabilized with ethanol, and reaction buffer comprising NAD<sup>+</sup> (grade V, Sigma-Aldrich Munich, Germany) and activator oligo (GGAATTCC) (Grube et al., 1991), dissolved in mM NaCl at 1mg/ml) was added followed by a post-fixation of the cells with paraformaldehyde. Then primary antibody (mouse monoclonal antibody 10H (purified as described previously, from culture supernatant of 10H hybridoma cells (Kawamitsu et al., 1984); kind gift of M. Miwa and T. Sugimura, Tokyo, Japan) using a protein-A column

chromatography kit (Pfeiffer et al., 1999)) directed against poly[ADP-ribose]) and fluorescent secondary antibody (Alexa Fluor® 488 goat anti-mouse secondary antibody was from Molecular Probes, Paisley, UK) incubation was performed with appropriate washing steps and finally flow cytometric analysis of immunofluorescence intensity.

**Statistical analysis:**

Each sample was analyzed in duplicate and the data were expressed as the mean value. A base-10 logarithmic transformation was applied to PARP activity and zinc concentration. We examined the association between cellular PARP activity and zinc concentration, as well as PARP activity as function of age by a correlation analysis using GraphPad InStat 3.

## Results

### Changes in plasma zinc concentrations after zinc supplementation

Oral supplementation of probands with 10 mg zinc aspartate for 7 weeks resulted in increased plasma zinc concentration in 41.4% of the donors we studied. By contrast, plasma zinc concentration decreased in 24.1%. In 34.5% there was no change in zinc concentration before and after zinc supplementation as shown in **Table 1**. Such differential effects could be observed in all three countries.

### Cellular poly(ADP-ribosylation) as function of plasma zinc concentration

Because of the frequent negative changes in plasma zinc concentrations after zinc supplementation, it appeared inappropriate to categorize the results of PARP activity measurements as “before” and “after” zinc supplementation but instead we related PARP activity to the actual plasma zinc concentration that prevailed in the sample. The effects of zinc on cellular poly(ADP-ribosylation) capacity are shown in **Figure 1**. The amount of poly(ADP-ribosylation) was positively and significantly correlated with higher cellular zinc concentrations ( $p < 0.05$ ). There were differences concerning poly(ADP-ribosylation) as function of plasma zinc concentration between Greece, Italy and Poland. As individual country, the Italian probands showed the only significant correlation ( $p < 0.02$ ) between PARP activity and zinc concentration compared to the other countries.

The Greek showed a non-significant tendency for PARP activity to be correlated with zinc concentration. In contrast, the samples from Poland did not show any positive correlation between PARP activity formation and zinc concentration. In order to highlight the effect of the change in plasma zinc concentration on the associated change in poly(ADP-ribosyl)ation capacity in individual donors, the changes (in percent, respectively) in these two parameters are plotted against each other in **Figure 2**. A significant positive correlation was observed ( $p < 0.05$ ).

### **Cellular poly(ADP-ribosyl)ation as function of donor age**

The results obtained by flow cytometry analysis revealed a tendency towards decreased poly(ADP-ribosyl)ation capacity with increasing donor age, as is illustrated in **Figure 3**, although this tendency did not reach statistical significance ( $p < 0.5$ ). However, a significant reduction in poly(ADP-ribosyl)ation capacity with donor age was observed in the PBMC of the Italian samples ( $p < 0.05$ ). The data from the two other countries analyzed separately failed to show a significant decline in poly(ADP-ribosyl)ation capacity as function of donor age ( $p < 0.5$ ).

## Discussion

The aim of the ZINCAGE project was to clarify the role of zinc on different biological and biochemical mechanisms that are involved in aging process. In this context, we focused on the link between PARP-1 activity and plasma zinc concentration, as well as PARP-1 activity as function of donor age.

We observed a positive correlation between poly(ADP-ribosyl)ation capacity in PBMC and plasma zinc concentration in the total population of probands we could analyze (**Figures 1 & 2**). Among the three countries there were differences concerning this positive correlation. The Italian probands showed a significant correlation between cellular poly(ADP-ribosyl)ation and zinc concentration, but not those from Poland, whereas the Greek subjects showed a non-significant trend towards a positive correlation. This difference can easily be explained by the much wider range of zinc concentrations covered by the Italian samples (**Figure 1**) thus providing better statistical power.

One explanation for the positive correlation between poly(ADP-ribosyl)ation capacity in PBMC and plasma zinc concentration observed in the total population (**Figures 1 & 2**), and in the Italian samples in particular, is that the higher availability of zinc in plasma should lead to higher intracellular concentrations thus guaranteeing full supply of zinc ions to zinc-binding proteins. This would include PARP-1, a zinc finger protein where zinc is essential for the binding to broken DNA. It has been shown that the zinc fingers of PARP-1 directly mediate the recognition of DNA strand breaks and thus enable enzyme activation (Mazen et al., 1989). It might well be that limited intracellular availability of zinc leads to

restricted PARP activity, and that such a deficit can be corrected by zinc supplementation. An alternative explanation is the following: It may be that due to the oxidative stress, which is known to increase with age (Mocchegiani et al., 2006), PARP-1 undergoes oxidative protein damage and is no longer fully active. Zinc supplementation can lead to improved cellular redox status (Hao and Maret, 2005) and thus antagonizes ROS production which can protect PARP-1 against oxidative damage.

Since the 1990s it is known that PARP is involved in the aging process. On the one hand cellular poly(ADP-ribosyl)ation correlates positively with species-specific life span in mammals (Grube and Burkle, 1992; Pero et al., 1985) and with human longevity (Muiras et al., 1998). On the other hand, poly(ADP-ribosyl)ation capacity decreases with donor age (Chevanne et al., 2007; Grube and Burkle, 1992). Although the age range (57-85 years) of the probands in the present study was far from covering the whole human life span, we did observe a significant decline of poly(ADP-ribosyl)ation capacity with donor age in the Italian probands.

Aging is clearly associated with genetic instability (Slagboom and Vijg, 1989), which is likely to contribute to cellular dysfunction, cellular senescence, malignant transformation or cell death. An impressive body of data has accumulated to show that PARP-1 facilitates DNA repair and antagonizes genomic instability in cells under genotoxic stress (Burkle, 2006). During the aging process there is a change in both factors: Cellular poly(ADP-ribosyl)ation capacity is decreasing (Grube and Burkle, 1992), as is the availability of free zinc (Mocchegiani et al.,

2006). Our present data demonstrate that zinc supplementation in elderly people can increase the cellular poly(ADP-ribosyl)ation capacity of their PBMC. We speculate that this may help maintain integrity and stability of the genome more efficiently and so contribute to an extension of healthspan.

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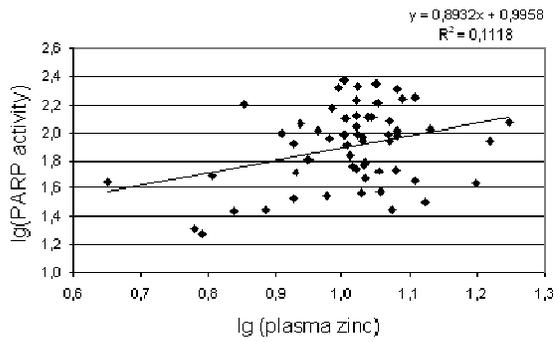
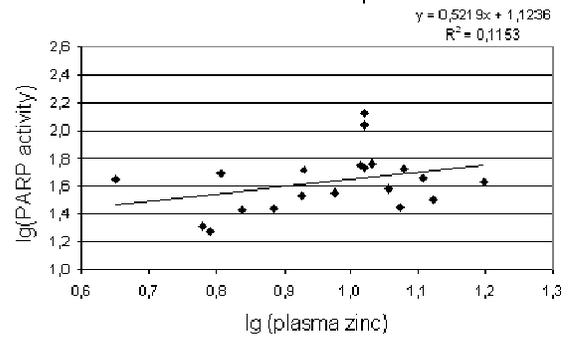
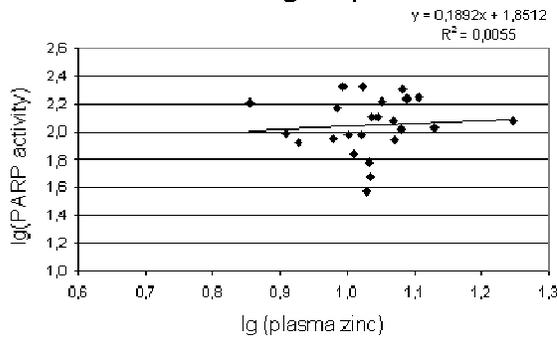
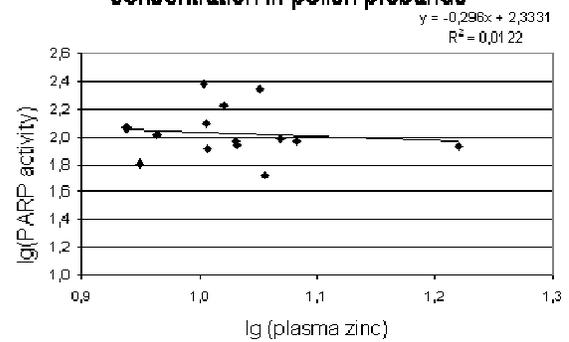
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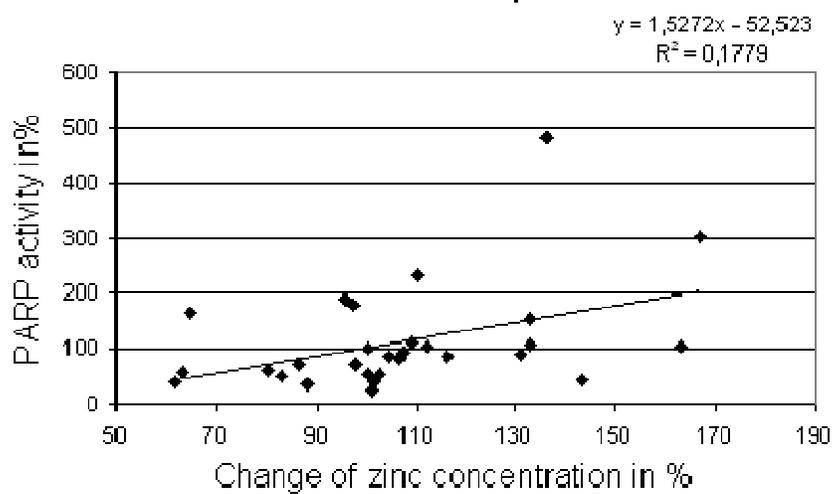
**Legend to figures**

**FIGURE 1:** Cellular poly(ADP-ribosyl)ation capacity as a function of plasma zinc concentration in PBMC from n=29 healthy old subjects. Poly(ADP-ribosyl)ation capacity and plasma zinc concentration was measured by a flow cytometry-based immuno assay and by induction coupled plasma mass spectrometry (ICP-MS), respectively. All scales are logarithmic. Each proband is represented by two data points, *i.e.* one before and one after zinc supplementation. A synopsis of all data revealed a significant positive correlation,  $p < 0.05$ . In separate analyses of the three countries we could observe a significant correlation only in the Italian subjects ( $p < 0.02$ ) but not in the two other countries ( $p < 0.5$ ).

**FIGURE 2:** Changes in PARP-1 activity (in %) upon 7-week zinc supplementation plotted as a function of changes in plasma zinc concentration (in %). Each data point represents one proband. The original data set is the same as in Figure 1A.

**FIGURE 3:** Cellular poly(ADP-ribosyl)ation capacity as function of donor age in PBMC from n=29 healthy old subjects. Poly(ADP-ribosyl)ation capacity was measured by a flow cytometry based immuno assay. A synopsis of all data showed a tendency but no statistical significance ( $p < 0.5$ ). A statistically significant negative correlation was observed in the Italian subjects ( $p < 0.05$ ), but not in Greek or Polish subjects ( $p < 0.5$ ).

**PARP activity as function of zinc concentration in all probands****PARP activity as function of zinc concentration in italian probands****PARP activity as function of zinc concentration in greek probands****PARP activity as function of zinc concentration in polish probands**

**PARP activity as function of zinc concentration in all probands**



**TABLE 1:** Changes in plasma zinc concentration in the study participants before zinc supplementation (bs) and after zinc supplementation (Z). Ten mg zinc per day was applied for 7 weeks Data are mean values.

Country	Plasma zinc concentration [μM] bs	Plasma zinc concentration [μM] Z	Δ zinc	Δ zinc in %
Italy	6.19	10.34	4.15	67.04
	7.68	10.48	2.80	36.46
	6.40	8.53	2.13	33.28
	11.86	15.78	3.92	33.05
	11.39	13.26	1.87	16.42
	12.00	12.80	0.80	6.67
	10.51	10.79	0.28	2.66
	10.48	8.43	-2.05	-19.56
	6.89	4.47	-2.42	-35.12
	9.49	6.01	-3.48	-36.67
Greece	7.14	10.24	3.10	43.42
	12.06	13.53	1.47	12.19
	9.57	10.55	0.98	10.24
	10.93	11.74	0.81	7.41
	8.09	8.45	0.36	4.50
	10.54	10.69	0.15	1.42
	9.89	9.69	-0.20	-2.02
	11.76	11.26	-0.50	-4.25
	12.29	10.82	-1.47	-11.96

	12.80	11.09	-1.71	-13.36
	12.09	10.06	-2.03	-16.79
	17.64	10.85	-6.79	-38.49
Poland	10.16	16.59	6.43	63.29
	9.21	12.09	2.88	31.27
	10.75	11.71	0.96	8.93
	10.47	10.73	0.26	2.48
	11.24	11.34	0.10	0.89
	10.09	10.12	0.03	0.30
	8.91	8.68	-0.23	-2.58