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AGE-RELATED INFLUENCE OF PIRACETAM ON MITOTIC INDEX AND NUMBER OF SILVER-STAINED NUCLEOLUS ORGANIZER REGIONS

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Abstract — Mitotic indices and the number of silver-positive nucleolus organizer regions (AgNORs) were scored in phytohemagglutinin-stimulated cultures of peripheral lymphocytes from two age groups of females ($\bar{x}$ = 23.1 and 84.0 yr, respectively) under the influence of Piracetam (2-oxo-pyrrolidine-1-acetamid; Nootropil®, Reg. No. 17051) and in simultaneously set up control cultures without Piracetam addition. Piracetam concentrations of 10, 14 and 16 mg/ml culture medium produced a highly significant, decreasing effect on both parameters tested, without an age-related difference. Lower Piracetam concentrations (2 and 4 mg/ml culture medium) showed a depressant effect on some of the cultures only; but, on average, there was a rather equal, significant, dose-dependent, linear decrease of the mitotic indices of both age groups, whereas the suppressive effect on the number of AgNORs was significant in cultures from the young females only.

In metaphase chromosomes only those rRNA gene cistrons (= nucleolus organizer regions, NORs) become visible by silver staining of NOR-associated proteins that were functionally active during the preceding interphase (= AgNORs; Miller, Dev, Tantravahi & Miller, 1976). Therefore, the number of AgNORs is considered to be a valuable criterion for cellular activity (Ploton, Menager, Jeannesson, Himber, Pigeon & Adnet, 1986). Piracetam (2-oxo-pyrrolidine-1-acetamid; Nootropil®, Reg. No. 17051)[‖] is a nootropic drug with a wide range of indications in geriatrics which has been shown to activate various steps of cellular metabolism not only in brain cells but other cell systems as well. Therefore, the effect of Piracetam on mitotic index and the number of AgNORs has been studied in phytohemagglutinin-stimulated, peripheral lymphocyte cultures. Since mitotic activity and the number of AgNORs found in this system depend, besides other factors, on the blood donor's age (Hefton, Darlington, Casazza & Weksler, 1980; Denton, Liem, Cheng & Barett, 1981), we investigated lymphocytes of females of two age groups.

EXPERIMENTAL PROCEDURES

Blood donors and lymphocyte cultures

The effect of Piracetam (2-oxo-pyrrolidine-1-acetamid, Nootropil®) on mitotic activity and the number of silver-stained NORs (= AgNORs) was determined after 72 h cultures of peripheral lymphocytes of two age groups of females (young: 17–27 yr, $\bar{x}$ = 23.1 yr; old: 80–88 yr, $\bar{x}$ = 84 yr).

The probands were selected after examination in the Outpatient Department of the Second Department of Internal Medicine, University of Vienna. All females of the old group came because of an imminent cataract operation; they were accepted for the study when examination revealed no other disturbance than hypertension and/or cardiopathy with appropriate treatment. The young females were suffering from hypotonia, neurasthenia, varicosis or onychomycosis — without treatment at the time of blood sampling. Blood was always taken before the X-ray examination of the chest. None of the probands was a smoker or

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Table 1. Young age group. Individual data and results of descriptive statistical analysis

Proband	Piracetam concentration mg/ml culture medium	MI	Chi^2 (DF = 1)	*P*	AgNORs/ metaphase *n* = 50 mean ± S.D.	*t* (DF = 98)	*P*
Subunit 1							
1	0	133			5.27 ± 1.09		
	2	85	9.54 (=0 vs 2)	<0.01	4.13 ± 1.25	5.30 (=0 vs 2)	<0.001
	4	62	47.16 (=0 vs 4)	<0.001	4.25 ± 1.45	3.77 (=0 vs 4)	<0.001
			6.70 (=2 vs 4)	<0.01		0.73 (2 vs 4)	n.s.
2	0	38			5.92 ± 1.51		
	2	32	0.50	n.s.	5.60 ± 1.30	1.04	n.s.
	4	14	10.80	0.001	3.85 ± 1.19	7.59	<0.001
			6.89	<0.01		7.10	<0.001
3	0	77			6.44 ± 1.11		
	2	61	1.74	n.s.	5.98 ± 1.17	2.02	<0.05
	4	46	7.36	<0.01	4.24 ± 1.14	9.35	<0.001
			3.99	<0.05		7.21	<0.001
4	0	143			6.92 ± 0.99		
	2	100	6.79	<0.01	6.30 ± 1.11	2.95	<0.01
	4	40	53.23	<0.001	5.56 ± 1.15	6.60	<0.001
			24.10	<0.001		3.39	0.001
5	0	139			6.74 ± 1.32		
	2	83	12.70	<0.001	6.70 ± 1.52	—	n.s.
	4	81	13.66	<0.001	4.50 ± 1.31	8.35	<0.001
			0.02	n.s.		7.76	<0.001
Subunit 2							
6	0	47			7.70 ± 0.99		
	10	18	12.50	<0.001	6.33 ± 1.82	4.91	<0.001
7	0	62			6.78 ± 1.96		
	10	38	5.49	<0.05	4.66 ± 1.47	6.12	<0.001
8	0	102			5.24 ± 2.08		
	14	58	11.21	<0.001	3.88 ± 1.99	3.51	<0.001
9	0	101			4.96 ± 1.67		
	14	69	5.55	<0.05	3.94 ± 1.17	3.54	<0.001
10	0	52			6.50 ± 1.09		
	16	14	21.19	<0.001	4.47 ± 1.01	9.80	<0.001
11	0	46			7.80 ± 0.99		
	16	31	2.80	n.s.	6.16 ± 2.10	5.34	<0.001

MI = Mitotic index (for definition see text); DF = degree of freedom; AgNORs = number of silver-positive nucleolus organizing regions.

alcoholic, none of the young females took oral contraceptives.

Both age groups were subdivided into two treatment groups: five subjects (No. 1 – 5 = subunit 1) were tested for Piracetam concentrations of 2 and 4 mg/ml culture medium and six subjects (No. 6 – 11 = subunit 2) for 10, 14 and 16 mg/ml culture medium. (Numbers of subjects and Piracetam concentrations were chosen after some preliminary experiments had shown that a concentration of 2 mg/ml culture medium might cause a weak influence only and that 20 mg/ml cause a very poor culture growth.) Piracetam was taken from commercial 15 ml Nootropil® ampoules (used in human medicine) containing 3 g Piracetam in a stable aqueous solution (Reg. No. 17051). Control

Table 2. Old age group. Individual data and results of descriptive statistical analysis

Proband	Piracetam concentration mg/ml culture medium	MI	Chi^2 (DF = 1)	*P*	AgNORs/ metaphase *n* = 50 mean ± S.D.	*t* (DF = 98)	*P*
Subunit 1							
1	0	43			4.25 ± 1.54		
	2	46	0.19	n.s.	4.10 ± 1.56	0.64	n.s.
			(=0 vs 2)			(=0 vs 2)	
	4	24	5.21	<0.05	4.39 ± 1.61	0.32	n.s.
			(=0 vs 4)			(=0 vs 4)	
			6.68	<0.01		0.93	n.s.
			(=2 vs 4)			(=2 vs 4)	
2	0	142			4.92 ± 1.27		
	2	71	21.41	<0.001	4.69 ± 1.13	0.42	n.s.
	4	48	42.55	<0.001	5.02 ± 1.31	0.39	n.s.
			4.20	<0.05		0.81	n.s.
3	0	54			6.46 ± 1.74		
	2	40	1.99	n.s.	6.18 ± 1.17	1.0	n.s.
	4	20	15.07	<0.001	5.60 ± 1.63	2.64	<0.01
			6.48	<0.05		2.10	<0.05
4	0	114			6.28 ± 1.31		
	2	60	15.43	<0.001	6.40 ± 1.14	0.41	n.s.
	4	17	67.55	<0.001	5.30 ± 1.44	3.62	<0.001
			23.13	<0.001		4.22	<0.001
5	0	69			7.78 ± 1.17		
	2	52	2.25	n.s.	6.98 ± 1.45	2.96	<0.01
	4	35	10.57	<0.01	5.92 ± 1.44	7.01	<0.001
			6.37	<0.05		3.67	<0.001
Subunit 2							
6	0	41			6.09 ± 1.65		
	10	23	4.91	<0.05	4.80 ± 1.41	5.08	<0.001
7	0	94			5.82 ± 1.65		
	10	17	50.67	<0.001	3.98 ± 1.04	6.67	<0.001
8	0	49			4.24 ± 1.09		
	14	21	10.82	<0.001	3.68 ± 1.11	3.08	<0.01
9	0	79			5.66 ± 1.23		
	14	6	60.21	<0.001	3.79 ± 1.15	8.11	<0.001
10	0	75			5.68 ± 1.26		
	16	6	56.55	<0.001	4.00 ± 1.26	6.75	<0.001
11	0	29			7.12 ± 1.21		
	16	6	14.86	<0.001	4.42 ± 1.40	10.99	<0.001

For abbreviations see footnote to Table 1.

cultures (= 0 mg Piracetam) and Piracetam cultures were always set up simultaneously with aliquots of leukocyte/plasma suspension gained from 20 ml blood. The medium used was RPMI 1640 (Gibco) containing 16.7% bovine fetal calf serum, 82.6 units/ml penicillin and 82.6 μg streptomycin. Cultures were stimulated by 0.2 ml phytohemagglutinin (PHA; HA 15, Wellcome). The final volume was 10 ml per flask. After 70 h culture time 0.1 ml colcemid (Gibco) was added, resulting in a final concentration of 0.1 μg/ml.

Chromosomal analysis

Chromosomes were prepared by the flame-dried technique, using 0.07 M KCl as hypotonic solution and methanol/glacial acid (3 : 2) as fixative. Mitotic indices were derived from Giemsa-stained slides by

Table 3. Age group comparison for the control data (=0 mg Piracetam; *t*-test)

		Young	Old	*P*
Mitotic index	$\bar{x}$	85.5	71.7	0.40
	S.D.	40.0	34.2	
	n	11	11	
Number of AgNORs	$\bar{x}$	6.38	5.85	0.24
	S.D.	0.97	1.09	
	n	11	11	

$\bar{x}$ = mean; S.D. = standard deviation; *n* = sample number; *P* = tail probability; AgNORs = silver-positive nucleolar organizing regions.

scoring the number of mitoses: 1000 interphase nuclei. Silver staining was performed according to the one-step method described by Howell & Black (1980). Briefly, a solution consisting of 1 g gelatine, 50 ml distilled water and 0.5 ml pure formic acid was mixed with 5 g/10 ml silver nitrate solution in the proportion 4 : 2 (on the slides). After 5 min incubation at room temperature and 80 – 90 min at 70°C the slides were washed with distilled water, air-dried and mounted.

Fifty metaphases with all 10 acrocentric chromosomes present were scored to establish the number of silver-positive acrocentrics (= AgNORs). All results were gained in a blind manner.

Statistical anlayses

The control values (0 mg Piracetam), mitotic indices and numbers of AgNORs, of the two age groups were compared by Student's *t*-test. The effects of the various Piracetam concentrations per proband were evaluated descriptively by means of the Chi-square test (mitotic indices) and *t*-tests (number of AgNORs). Analysis of variance with repeated measures was used to test the dosage effect within each age group, with modification of Huynh – Feldt for subunit 1 (0, 2 and 4 mg). In addition, the dose factor was broken into orthogonal linear and quadratic components. To compare the effects of age and dosage together a split-plot analysis of variance was performed, whereby age was the between-factor and dosage the within-factor, an interaction term was included. If necessary, the Huynh – Feldt correction was applied. The *t*-test was used to compare the effects of 4 mg Piracetam between age groups. Correlation coefficients were determined to test associations between mitotic indices and number of AgNORs.

RESULTS

All the individual data and the results of descriptive statistical comparisons pro proband are given in Table 1 (young) and Table 2 (old), and the results of group-comparisons in Tables 3, 4 and 5.

In the control cultures (0 mg Piracetam) the young probands had a higher mean mitotic index and a higher mean number of AgNORs than the old ones but the standard deviations were high and the differences proved to be not significant (Table 3).

As to the Piracetam effects on mitotic indices the young probands showed a statistically remarkable decrease for all but three cultures (two 2 mg cultures, one 16 mg culture) and the old probands had a significant decrease for all but three 2 mg cultures (Tables 1 and 2, respectively).

Considering subunit 1 (0, 2 and 4 mg) there is an approximately linear decrease with increasing dosage in both age groups (Table 4a). This highly significant dosage effect is also documented by the split-plot analysis of variance (Table 4b). Highly significant decreases could be established for both subunit 2 (Table 5a) and in the split-plot analysis of variance (Table 5b), but no age effect could be established.

As to the number of AgNORs, young probands showed a significant decrease in all 4 mg cultures and in three of the five 2 mg cultures (Table 1), old probands, on the other hand, showed no significant effect in two of the five 4 mg cultures and four of the five 2 mg cultures (Table 2). A significant, dose-dependent decrease could be documented for the young probands only (Table 4a). This difference between age groups is caused by the 0 vs 4 mg changes, the comparison of which revealed a *P*-value of 0.048 (*t*-test), and is expressed by an interaction *P*-value of 0.07 (Table 4b).

The high Piracetam concentrations (10, 14 and 16 mg) caused highly significant decreases in both age groups (Tables 1, 2, 5a and 5b), with no age effect and no interaction age – dosage.

There does not exist any correlation between mitotic indices and the number of AgNORs (young: $r = -0.38$; old: $r = -0.13$; young + old: $r = 0.19$).

DISCUSSION

Piracetam is a nootropic drug which exhibits particular affinity for the grey matter and various nuclei of the brain (Ostrowski & Keil, 1978). The well-documented Piracetam-induced improvement

Table 4a. Dosage comparison within each age group for subunit 1

	Age group		Piracetam concentration (mg/ml culture medium) 0	2	4	P	P^L	P^Q
Mitotic index	Young (n = 5)	$\bar{x}$	106.0	72.2	48.6	0.003	0.02	0.48
		S.D.	46.5	26.4	25.0			
	Old (n = 5)	$\bar{x}$	84.4	53.8	28.8	0.03	0.03	0.67
		S.D.	42.0	12.1	12.7			
Number of AgNORs	Young (n = 5)	$\bar{x}$	6.24	5.74	4.48	0.01	0.003	0.26
		S.D.	0.71	0.99	0.65			
	Old (n = 5)	$\bar{x}$	5.94	5.65	5.24	0.12	0.13	0.75
		S.D.	1.37	1.24	0.58			

P = global comparison; P^L = linear component; P^Q = square component; n = sample number; $\bar{x}$ = mean; S.D. = standard deviation; AgNORs = silver-positive nucleolar organizer regions.

Table 4b. Results of split-plot-analysis of variance for subunit 1

	Age	Dosage effect	Interaction
Mitotic index	0.26	0.0001	0.97
Number of AgNORs	0.83	0.0004	0.07

of an age-dependent or pathological decline of cerebral functions relates to an activation of cerebral energy metabolism (Gobert & Temmermann, 1973), to a stimulating effect on the neuron respiratory chains (Woelk & Peiler-Ichikawa, 1978), to an increased biosynthesis of phospholipids (Rochus & Reuse, 1976), RNA and polyribosomes (Gobert & Temmermann, 1973) and protein (Platt, Hering & Hering, 1974), and such an increase in protein synthesis should be combined with an increase in nucleolar activity (see below). There are only a few studies dealing with the effect of Piracetam on other tissues than brain: an increase of cAMP has been demonstrated for gut cells of guinea-pigs (Weth, 1981) and there are effects on sickle and diabetic erythrocytes, i.e. enhancement of the deformibility of the cell membrane (Targino de Aranjo & Nero, 1980; Nalbandian & Henry, 1981), platelets — resulting in a platelet antiaggregant activity (Barnhart, Barmatoski & Penner, 1980) and neutrophils — restoring ineffective to absent phagocytic activity and/or respiratory burst intensity (Nalbandian, 1982). Therefore, an effect on peripheral lymphocytes seemed to be possible as well. In human medicine daily doses of 1.2 – 24.0 g Piracetam are administered. Blood levels of 1 – 2 mM (mol. wt = 142) may be achieved by administration of 10 g Piracetam daily (Barnhart, Walz, Penner & Bauer, 1981). That is, even after administration of 24 g daily the Piracetam-concentration in blood is far below the concentrations used in our *in vitro* studies. But, as to our knowledge, nothing is known about binding and uptake of Piracetam by T-lymphocytes).

The phytohemagglutinin (PHA)-stimulated peripheral lymphocyte culture is a well-established *in vitro* system which mimics the *in vivo* antigen response of T-lymphocytes with induction of transformation and mitosis. The main morphological feature of transformation is the formation of large nucleoli that were built up by the fusion of two or more activated nucleolus organizer regions (AgNORs; "satellite association"; Ferguson-Smith & Handmaker, 1961; Schwarzacher & Wachter, 1983). In the human genome the NORs are localized in the secondary constrictions (= stalks) of the five pairs of acrocentric chromosomes (D13, D14, D15, G21, G22). NORs possess the ribosomal RNA (rRNA) genes, approximately 40 transcriptional units per NOR (Lewin, 1980). Active NORs were transcribed by polymerase I (Perry, 1976).

In metaphase chromosome spreads only those NORs that were transcribing in the preceding interphase become visible by silver staining of the NOR-associated proteins (= AgNORs) (Miller *et al.*, 1976) the nature of which is not fully understood (Williams, Kleinschmidt, Khrone & Franke, 1982; Ochs & Busch, 1984; Olson & Thompson, 1983;

Table 5a. Dosage comparison within each age group for subunit 2

	Age group		Piracetam concentration (mg/ml culture medium) 0	10 – 16	*P*
Mitotic index	Young	$\bar{x}$	68.3	38.0	0.0008
	(n = 6)	S.D.	26.3	21.8	
	Old	$\bar{x}$	61.2	13.2	0.008
	(n = 6)	S.D.	25.2	8.1	
Number of AnNORs	Young	$\bar{x}$	6.50	4.91	0.0003
	(n = 6)	S.D.	1.20	1.08	
	Old	$\bar{x}$	5.77	4.11	0.002
	(n = 6)	S.D.	0.93	0.42	

For abbreviations please see Table 3.

Table 5b. Results of split-plot-analysis of variance for subunit 2

	Age	Dosage effect	Interaction
Mitotic Index	0.17	0.0001	0.17
Number of AgNORs	0.17	<0.0001	0.85

For abbreviations please see Table 3.

Hernandez-Verdun, Derenzini & Bouteille, 1984). Therefore, the number of AgNORs appears to reflect cell activity, since rRNA is of vital significance for protein synthesis.

The number of AgNORs varies markedly between different cells of one and the same culture, depends on donor age (Denton *et al.*, 1981), as it has been demonstrated by our results, and culture conditions, e.g. fetal calf serum concentration (De Capoa, Marlekaj, Baldini, Archidiacono & Rocchi, 1985), and may be influenced by environment, e.g. substances produced by cancer cells (Cheng, Denton, Liem & Elliot, 1981; Kivi & Mikelsaar, 1985). Considering lymphocyte cultures and satellite associations (SA) whose frequency usually is positively correlated with that of AgNORs (Di Lernia, 1980), variations in SA have been induced by X-rays and Dioxin (Di Lernia, Crimaudo & Paccetti, 1982), oral contraceptives (Li & Zhou, 1983), thyroid hormone (Nilsson, Hansson & Nilsson, 1975), etc.

With regard to the well-documented metabolic effects of Piracetam — particularly concerning protein metabolism — an increase in the number of AgNORs and/or mitotic indices should be found in Piracetam cultures. Therefore, the observed contrary effect has to be explained by the specific conditions of the *in vitro*-system used. First of all, a competitive interaction between PHA, its binding to membrane receptors and/or interaction with one of the numerous secondary effects (Tollefsbol & Cohen, 1986), has to be considered. And indeed, there is a decrease in mitotic indices as well as numbers of AgNORs in all cultures with high Piracetam concentrations (subunit 2). But, in cultures with low Piracetam concentrations (subunit 1) five cultures showed a significant decrease in mitotic index without a significant decrease in the number of AgNORs (Tables 1 and 2), although an increased rRNA-synthesis is considered to be a prerequisite of cell division (Ahern & Kay, 1975).

Comparing the Piracetam effects found in the two age groups, a higher sensitivity could be demonstrated for the young probands which became apparent in the low-dosage experiments. One might speculate that the higher PHA-induced activation of cellular metabolism in the cells from young probands might be the non-specific cause for the higher vulnerability to Piracetam. But, a specific, depressant mechanism might exist as well and in this case elucidation of the underlying mechanism could be of general significance to geriatric research.

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