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Interaction of chlorpromazine, fluphenazine and trifluoperazine with ocular and synthetic melanin *in vitro*

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The aim of this study was to examine *in vitro* the binding capacity of three phenothiazine derivatives – chlorpromazine, fluphenazine and trifluoperazine – causing adverse effects in the eye structures, to natural melanin isolated from pig eyes as well as to synthetic DOPA-melanin used as a model polymer. The amount of drug bound to melanin was determined by UV spectrophotometry. The analysis of results for the kinetics of drug-melanin complex formation showed that the amount of drug bound to melanin increases with increasing initial drug concentration and longer incubation time, attaining an equilibrium state after about 24 h. Binding parameters, i.e. the number of binding sites (n) and association constants (K), were determined on the basis of Scatchard plots. For neuroleptic-ocular melanin and neuroleptic-DOPA-melanin complexes two classes of independent binding sites were found, with association constants $K_1 \sim 10^4$ and $K_2 \sim 10^2$ M^{-1} for chlorpromazine and fluphenazine complexes, and $K_1 \sim 10^5$ and $K_2 \sim 10^3$ M^{-1} for trifluoperazine complexes. The numbers of strong (n_1) and weak (n_2) binding sites indicate lower affinity of the drugs examined to ocular melanin compared with DOPA-melanin. The ability of chlorpromazine, fluphenazine and trifluoperazine to interact with melanin, especially the ocular melanin, *in vitro* is discussed in relation to the ocular toxicity of these drugs *in vivo*.

1. Introduction

Chlorpromazine – an aliphatic phenothiazine derivative – and fluphenazine and trifluoperazine – piperazine phenothiazine derivatives – are neuropsychiatric agents of the neuroleptic group (Baldessarini 1996; Delgado and Remers 1998). Chlorpromazine was the earliest phenothiazine derivative introduced into therapy, having significant sedative and hypotensive properties. Because fluphenazine and trifluoperazine have both 2-trifluoromethyl and piperazine groups, they are potent antipsychotic agents with low sedative and hypotensive effects (Delgado and Remers 1998). Phenothiazine derivatives are widely used in the treatment of psychotic disorders including schizophrenia, psychosis, maniacal states and behaviour disturbances (Baldessarini 1996; Marques et al. 2004; Parfitt 2002; Walsh 2001). Moreover, trifluoperazine, as one of the most potent calmodulin inhibitors (intracellular mediator for calcium ions), induces an analgesic effect (Golbidi et al. 2002) and demonstrates fungicidal (Sharma et al. 2001) and antimycobacterial (Gadre et al. 1998) activity. Numerous side effects are due to phenothiazine administration, e.g. extrapyramidal side effects and resultant disorders including acute dystonia, a parkinsonism-like syndrome, akathisia and tardive dyskinesia (fluphenazine and trifluoperazine exert stronger extrapyramidal effects than chlorpromazine), neuroleptic malignant syndrome, ocular toxicity, and alteration of endocrine and metabolic functions (Baldessarini 1996; Marques et al. 2004; Parfitt 2002; Delgado and Remers 1998). Aliphatic derivatives exert stronger drug-induced ocular side effects than piperazine derivatives; however these effects can also appear with prolonged use of piperazine derivatives (Buszman and Różańska 2003a; Wolf et al. 1993). Therapy with phenothiazine derivatives can lead to corneal endothelial phototoxicity (Hull et al. 1982, 1983; Panzit et al. 2001; Hollander and Aldave 2004), and pigmentary changes of conjunctiva, sclera and lens (Panzit et al. 2001; Rennie 1993; Walsh 2001; Hollander and Aldave 2004). Moreover, chlorpromazine, fluphenazine and trifluoperazine can cause drug-induced degenerative retinopathy with histological, electrophysiological and symptomatological features similar to those of primary retinitis pigmentosa (Buszman and Różańska 2003a; Fornaro et al. 2002; Parfitt 2002; Toler 2005). Subjective symptoms of neuroleptic-induced retinopathy are as follows: decrease in vision, disturbances of dark adaptation, night blindness and central scotoma, and they generally precede abnormalities of the retina including degeneration and pigmentation. In serious cases this can even cause complete or very severe loss of vision (Fornaro et al. 2002; Rennie 1993; To 2000). The exact mechanism of phenothiazine retinal toxicity is still unknown. It is suggested that blockade of retinal dopamine receptors (localized mainly in the photoreceptor layer and retinal pigment epithelium – RPE cells), and drug binding to melanin granules in RPE cells (causing alterations of

retinal enzyme kinetics, loss of photoreceptors, RPE and choriocapillaris with damage to the rods and cones through inhibition of oxidative phosphorylation), can lead to drug-induced retinopathy (Fornaro et al. 2002; Toler 2004).

Melanin pigments are multifunctional polymers. The physiological function of melanin is to buffer against photochemical stress through absorption and dispersing UV radiation, sequestering metal ions and trapping free radicals and reactive oxygen species (Hu 2005; Nofsinger et al. 2002; Tolleson 2005). Biopolymer granules are able to bind many heterogeneous chemical compounds such as metal ions, organic amines and cyclic compounds (i.e. drugs). By inhibiting or significantly restricting drug access to cell receptors, they protect the organism against drug side effects. On the other hand, long-term exposure and slow release of drugs or their metabolites from bonds may build up high and long-lasting levels of noxious chemicals stored by melanin, which may cause degeneration in the melanin-containing cells (especially in the eye, ear, skin and brain) and secondary lesions in surrounding tissues (Hu et al. 2002; Larsson 1993; Mars and Larsson 1999).

The purpose of the studies was to examine *in vitro* the binding capacity of the phenothiazine derivatives chlorpromazine, fluphenazine and trifluoperazine to both natural and synthetic DOPA-melanin. To achieve this, the kinetics of drug-melanin complex formation were examined, and the number of independent binding sites and the association constants were also determined. Natural ocular melanin isolated from pig eyes was used with synthetic DOPA-melanin as a model polymer.

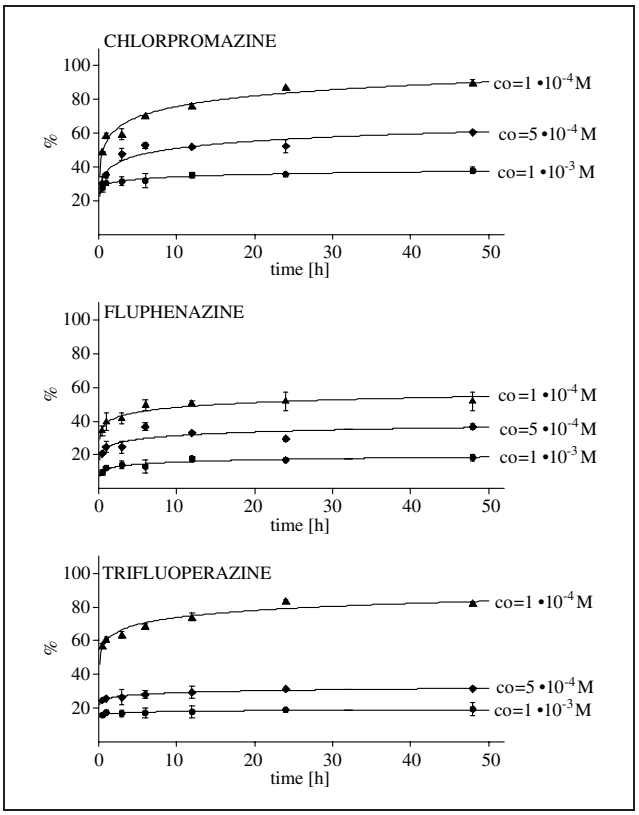

Fig. 1: Effect of incubation time and initial drug concentration (c_0) on amount of chlorpromazine, fluphenazine and trifluoperazine bound to DOPA-melanin (%).
Mean values ± SD from three independent experiments are presented. Points without error bars indicate that SD was less than the size of the symbol

2. Investigations and results

Kinetics of the formation of chlorpromazine, fluphenazine and trifluoperazine-melanin complexes shown as the relationship between the amount of drug bound to the polymer and the incubation time are presented in Fig. 1 for three initial drug concentrations (c_0). It is demonstrated that longer incubation time results in an increased amount of drug bound to synthetic melanin. On the basis of the results it may be concluded that the maximum time to achieve an equilibrium state is 24 h. Complex formation efficiency (the ratio of the amount of drug bound to DOPA-melanin and the amount of drug added to form the complex, expressed as %) decreased with increased initial drug concentration.

A relationship between the amount of chlorpromazine, fluphenazine and trifluoperazine bound to melanin after 24 h of incubation and the initial drug concentration for ocular melanin and for synthetic-DOPA melanin, as binding isotherms, is presented in Fig. 2A and Fig. 3A, respectively. It may be seen from the binding curves that the amount of drug bound to melanin increased with increased initial drug concentrations. In the concentration range studied (from 5×10^{-5} M to 3×10^{-3} M) the increase was 12-fold for chlorpromazine complexes with both ocular (0.0338 to 0.4189 μmol/mg) and synthetic (0.0478 to 0.5259 μmol/mg) melanin, and 6-fold for fluphenazine-melanin complexes (0.0274 to 0.1814 μmol/mg for ocular and 0.0359 to 0.2138 μmol/mg for synthetic melanin). In the case of trifluoperazine the increase was 3- and 2-fold respectively for drug-eye melanin

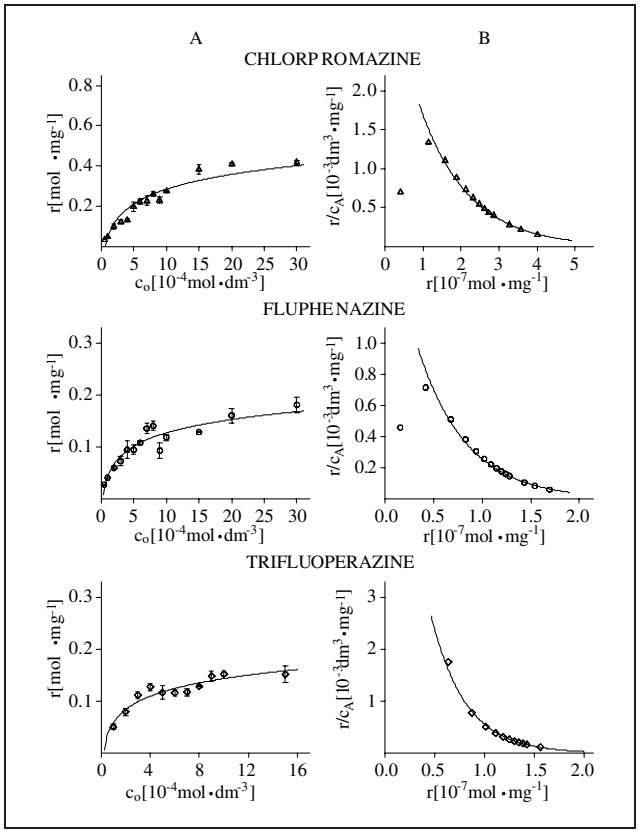

Fig. 2: Binding isotherms (A) and Scatchard plots (B) for chlorpromazine-ocular melanin, fluphenazine-ocular melanin and trifluoperazine-ocular melanin complexes. r – amount of drug bound to melanin; c_0 – initial drug concentration; c_A – concentration of unbound drug.
Mean values ± SD from three independent experiments are presented. Points without error bars indicate that SD was less than the size of the symbol

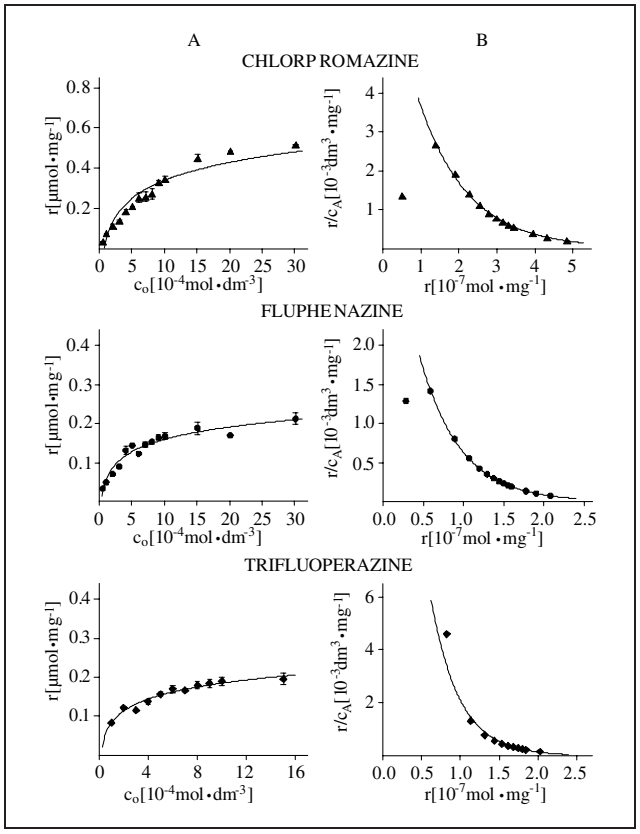

Fig. 3: Binding isotherms (A) and Scatchard plots (B) for chlorpromazine-DOPA-melanin, fluphenazine-DOPA-melanin and trifluoperazine-DOPA-melanin complexes. r – amount of drug bound to melanin; c_0 – initial drug concentration; c_A – concentration of unbound drug.
Mean values ± SD from three independent experiments are presented. Points without error bars indicate that SD was less than the size of the symbol

(0.0482 to 0.1516 μmol/mg) and drug-model melanin (0.0831 to 0.1915 μmol/mg) complexes. Moreover, it can be seen that the maximum amounts of chlorpromazine, fluphenazine and trifluoperazine bound to 1 mg of synthetic DOPA-melanin are 26, 18 and 29%, respectively, compared with the amounts of these drugs bound to natural melanin isolated from pig eyes.

The use of the Scatchard method can provide information on binding parameters, i.e. the association constants and the number of binding sites for drug-melanin complexes. For the complexes of chlorpromazine, fluphenazine and trifluoperazine with ocular and synthetic melanin analyzed, the Scatchard plots (Figs. 2B and 3B) are curvilinear with an upward concavity indicating that at least two classes of independent binding sites are involved in the formation of these complexes. Comparing the number of strong (n_1) and weak (n_2) binding sites (Table) it may be observed that weak binding sites (n_2) predominate: the ratio n_2/n_1 ranges from 1.2 to 1.8. The total number of binding sites ($n_1 + n_2$) is higher for drug-synthetic melanin complexes than for drug-ocular melanin complexes, indicating greater affinity of the phenothiazine derivatives tested to synthetic DOPA-melanin. Moreover, analysis of the total number of binding sites in the drug-melanin complexes examined indicates that trifluoperazine has the lowest affinity to ocular melanin *in vitro* ($n_1 + n_2 = 0.18$ μmol/mg), and chlorpromazine the highest ($n_1 + n_2 = 0.53$ μmol/mg). Similar observations can be noted for the phenothiazines-synthetic melanin complexes analyzed. At the same time, trifluoperazine-melanin complexes are characterized by higher stability ($K_1 \sim 10^5$ M^{-1}, $K_2 \sim 10^3$ M^{-1}) compared with chlorpromazine- and fluphenazine-melanin complexes ($K_1 \sim 10^4$ M^{-1}, $K_2 \sim 8–9 \times 10^2$ M^{-1}).

3. Discussion

The mechanism by which systemic medications cause ocular side effects remains mostly unknown. Probably a combination of different mechanisms is responsible for the various lesions (Fornaro et al. 2002; Moorthy and Valluri 1999). The potential for ocular toxicity typically stems from the underlying chemical properties of the drug rather than its pharmacological effect (Hollander and Aldave 2004). Intraocular structures are shielded from systemic toxins by the blood-aqueous barrier anteriorly and the blood-retinal barrier posteriorly. There are two barriers to the entry of compounds into the retina: the tight junctions of the retinal capillary endothelium and the tight junctions between the RPE cells. Lipophilic drugs (i.e. chlorpromazine, fluphenazine, trifluoperazine) are more likely to pass through these barriers into the eye than are hydrophilic drugs (Moorthy and Valluri 1999; Roberts 2002). These

Table: Binding parameters for chlorpromazine, fluphenazine and trifluoperazine complexes with ocular and synthetic melanin

Drug-melanin complex	Association constants K [M^{-1}]	Number of binding sites n [μmol drug/mg mel]
Chlorpromazine – ocular melanin	$K_1 = 1.24 \times 10^4$	$n_1 = 0.2054$
	$K_2 = 8.06 \times 10^2$	$n_2 = 0.3225$
		$n_1 + n_2 = 0.5279$
Chlorpromazine – synthetic melanin	$K_1 = 2.37 \times 10^4$	$n_1 = 0.2386$
	$K_2 = 9.21 \times 10^2$	$n_2 = 0.3798$
		$n_1 + n_2 = 0.6184$
Fluphenazine – ocular melanin	$K_1 = 1.52 \times 10^4$	$n_1 = 0.0852$
	$K_2 = 8.30 \times 10^2$	$n_2 = 0.1360$
		$n_1 + n_2 = 0.2212$
Fluphenazine – synthetic melanin	$K_1 = 2.60 \times 10^4$	$n_1 = 0.1042$
	$K_2 = 9.32 \times 10^2$	$n_2 = 0.1504$
		$n_1 + n_2 = 0.2546$
Trifluoperazine – ocular melanin	$K_1 = 1.16 \times 10^5$	$n_1 = 0.0671$
	$K_2 = 2.32 \times 10^3$	$n_2 = 0.1177$
		$n_1 + n_2 = 0.1848$
Trifluoperazine – synthetic melanin	$K_1 = 1.78 \times 10^5$	$n_1 = 0.1035$
	$K_2 = 2.80 \times 10^3$	$n_2 = 0.1254$
		$n_1 + n_2 = 0.2289$

natural ocular barriers and ocular structures may also act as drug depots and can play an important role in the pathogenesis of drug-induced ocular toxicity (Moorthy and Valluri 1999). Moreover, compounds that have either a tricyclic, heterocyclic or porphyrin ring structure and are incorporated into ocular tissues are potentially toxic agents in the eye (Roberts 2002).

The toxicity of the drug itself (depending on the dose and duration of therapy), whether administered locally or systemically and the binding of drug to melanin can be among the risk factors of the drug induced ocular side effects. Binding of the drug to melanin granules can alter the chemical, biological, photobiological and photochemical properties of both melanin and drug (Buszman 1994; Moorthy and Valluri 1999; Roberts 2002). In the eye, drugs may become trapped in one or more of the major ocular depots, i.e. cornea, lens, vitreous and melanin (ciliary pigment epithelium, RPE and melanocytes) (Moorthy and Valluri 1999). The pigmented cells are located close to the receptor cells, and melanin binding may be an important factor in the development of some drug-induced ocular lesions (Larsson 1993). In addition, slow release of drug from depots causes prolonged exposure of pigmented eye tissues to the toxicity of the drug and can lead to the development of many pathological lesions (Hu et al. 2002; Leblanc et al. 1998; Moorthy and Valluri 1999). Retinal lesions are one of the most dangerous alterations in the eye structures connected with the ocular toxicity of drugs, because they can lead to pigmentary retinopathy (toxic retinopathy) and in serious cases can even cause complete or severe loss of vision (Buszman and Różańska 2003b; Dayhaw-Barker 2002; To 2000). Neuroleptics – a heterogeneous group of pharmacological agents commonly used for the treatment of psychiatric disorders – can cause unwanted side effects, including eye and visual manifestations which can be severe and sometimes irreversible (Fornaro et al. 2002; Parfitt 2002).

In our studies, the binding capacity of three phenothiazine derivatives, chlorpromazine, fluphenazine and trifluoperazine, to natural melanin isolated from pig eyes and also to synthetic DOPA-melanin was demonstrated. Studies on the formation kinetics of the neuroleptic-melanin complexes examined showed that the amount of drug bound to melanin increased with a rise in initial drug concentration and with longer incubation time, attaining equilibrium after about 24 h.

An analysis of chlorpromazine, fluphenazine and trifluoperazine binding to ocular and DOPA-melanin by the use of Scatchard plots showed that at least two classes of independent binding sites are involved in the formation of these complexes: strong binding sites (n_1) with an association constant $K_1 \sim 10^4 - 10^5\ M^{-1}$ and weak binding sites (n_2) with K_2 about $1 \times 10^3\ M^{-1}$. The results obtained also show that trifluoperazine forms the most stable complexes with both ocular and synthetic melanin as compared with chlorpromazine- and fluphenazine-melanin complexes. The similar values of the association constants for ocular and DOPA-melanin complexes with the neuroleptics tested suggest that these two melanins are similar in their structure and physicochemical properties, which confirms the possibility of using synthetic DOPA-melanin as a model for drug-melanin interaction studies.

The analysis of the total number of binding sites for the phenothiazines examined indicates that trifluoperazine has the lowest affinity to both ocular and synthetic DOPA-melanin. Taking into account the total number of binding sites, the order of the drugs affinity to ocular and synthetic melanin would be: chlorpromazine $\gg$ fluphenazine $>$ trifluoperazine. This is probably one of the reasons for the greater tendency of chlorpromazine to induce undesirable ocular side effects than is shown by fluphenazine and trifluoperazine.

The ability of the phenothiazine derivatives analyzed to form complexes with melanin, especially ocular melanin isolated from pig eyes, *in vitro* may be one of the reasons for their ocular toxicity *in vivo*, as a result of their interaction with melanin in the pigmented structures of the eye.

4. Experimental

4.1. Chemical reagents

L-3,4-Dihydroxyphenylalanine (L-DOPA), chlorpromazine (hydrochloride), fluphenazine (dihydrochloride) and trifluoperazine (dihydrochloride) used in the studies were obtained from Sigma Chemical Co. The remaining chemicals were produced by P.P.H. POCh, Poland.

4.2. Synthetic DOPA-melanin

DOPA-melanin was obtained by oxidative polymerization of L-3,4-dihydroxyphenylalanine (L-DOPA) solution (1 mg/ml) in 0.067 M phosphate buffer at pH 8.0 according to the Binns method (Binns et al. 1970).

4.3. Isolation of ocular melanin

The pig eyes were dissected to separate the iris and choroid with the retinal pigment epithelium (RPE). The procedure of melanin isolation was performed according to the Persad method (Persad et al. 1986). From 182 pig eyes about 372 mg of ocular melanin was obtained.

4.4. Drug-melanin complex formation

Drug-melanin complexes were obtained by suspending 5 mg of ocular or synthetic melanin in 5 ml of chlorpromazine, fluphenazine and trifluoperazine solution. Three independent samples were prepared for each drug concentration and time of complex formation. A mixture of melanin and drug solution was incubated at room temperature and then filtered. Control samples, containing melanin suspended in water, were treated in the same way.

4.5. Determination of the amount of drug bound to melanin

The UV spectrophotometric method was used for quantitative determination of the drugs studied. Absorption spectra in the UV-VIS range were measured for aqueous solutions of the drugs. Analytical wavelengths (λ_{max}) for the compounds studied were chosen as follows: 255 nm for chlorpromazine and 257 nm for fluphenazine and trifluoperazine. The calculated values of the molar absorption coefficient ($\varepsilon_{\lambda max}$), 3.1×10^4 for chlorpromazine, 2.9×10^4 for fluphenazine and 2.9×10^4 for trifluoperazine, were used to estimate the amount of drug bound to the polymer. All spectrophotometric measurements were performed using a JASCO model V-530, UV-VIS spectrophotometer.

4.6. Kinetics of drug-melanin complex formation

Kinetics of formation of melanin complexes with chlorpromazine, fluphenazine and trifluoperazine were evaluated on the basis of the relationship between the amount of drug bound to the polymer (μmol/mg) and the time of complex formation. In the studies, the following initial drug concentrations were used: 1×10^{-4}, 5×10^{-4} and 1×10^{-3} M. Complex formation was studied at for 0.5, 1, 3, 6, 12, 24 and 48 h.

4.7. Binding parameters of drug-melanin complexes

The number of strong (n_1) and weak (n_2) binding sites and the association constants (K) of the ocular and synthetic melanin complexes with chlorpromazine, fluphenazine and trifluoperazine were calculated *via* Scatchard plots (Kalbitzer and Stehlik 1979). Experimental binding isotherms were used to construct these plots. They show the relationship between the amount of drug bound to melanin and its initial concentration after reaching an equilibrium state, i.e. after 24 h. The initial drug concentration was 5×10^{-5} M to 3×10^{-3} M for chlorpromazine and fluphenazine, and 5×10^{-5} M to $1 \cdot 5 \times 10^{-3}$ M for trifluoperazine.

4.8. Statistical analysis

In all experiments, mean values for three independent experiments ± standard deviations (S.D.) were calculated.

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