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LETTER TO THE EDITOR

Haloperidol Reduces the Activity of Complement and Induces the Anti-Inflammatory Transformation of Peritoneal Macrophages in Rats

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Abstract

It is known that psychotropic substances affect the immune system. Unfortunately, chronic antipsychotic administration causes side toxicological effects, associated with oxidative stress. The mechanisms of these effects are still unclear. We investigated the impact of sub-chronic administration of haloperidol (Hal) on parameters of innate immunity and related systems in healthy rats and compared them with Hal content. Hal administration (0.5 mg/kg, 3 weeks) resulted in two-fold decrease of the activity of the complement system and hemostasis. Hal content correlated with the activity of the complement ($r = -0.71$), phagocytic activity of peritoneal macrophages ($r = 0.78$), leukocyte elastase ($r = -0.71$) and glutathione-S-transferase activity ($r = -0.67$). Hal fully blocked in vitro PMA-induced iNOS expression in macrophages and changed their morphology to "anti-inflammatory" phenotype. The comparison of in vivo and in vitro data showed that Hal has a direct effect on phagocytic component of innate immunity and an indirect effect on leukocyte elastase and antioxidant enzymes. The results obtained in the present study indicated that Hal significantly affects homeostasis and causes a number of complex biological transformations.

Keywords Haloperidol · Innate immunity · Complement system · Macrophages · Hemostasis · Antioxidant system · Rats

Highlights

1. Haloperidol reduce the activity of the complement system and hemostasis in vivo
2. Haloperidol block iNOS expression in macrophages and change their shape in vitro
3. Haloperidol has an indirect effect to leukocyte elastase and antioxidant enzymes
4. Haloperidol significantly affect homeostasis

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Introduction

It is known that inflammation plays a significant role in mental disorders and that psychotropic substances have an immunotropic effects (Kaminska et al. 2001; Müller et al. 2015). Unfortunately, chronic antipsychotic administration causes side effects, associated with oxidative stress, which results in metabolic syndrome, tardive dyskinesia, agranulocytosis (Anderson et al. 2013; Gumulec et al. 2013). The mechanisms of these effects are still unclear and there are a few studies that directly compare antipsychotic's concentration in blood with immunity parameters. Typical antipsychotic haloperidol has been shown to increase serum levels of prolactin and corticosterone in rats. In addition, haloperidol-induced changes in corticosterone and prolactin levels reduce Mycobacterium-induced macrophage activation in vivo (Lourenço et al. 2005).

Therefore, our aim was to study the effect of subchronic administration of haloperidol (Hal) on certain parameters of innate immunity, hemostasis and antioxidant system of healthy rats, and to compare these parameters with Hal concentration in blood. The tasks were: to evaluate the effect of Hal on phagocytic activity of peritoneal macrophages, inducible NO-synthase (iNOS) expression level in these cells and leukocyte elastase (LE) activity in blood serum. As complement system (CS) is an important regulator of immunity, we have studied the effect of Hal on CS activity and associated hemostasis system. Glutathione is one of the main redox balance regulators. Glutathione-S-transferase (GT) enzyme uses reduced glutathione in reactions of conjugation and detoxication of xenobiotics. In addition, glutathione reductase (GR) enzyme provides pool support of reduced glutathione. Therefore in our research we have evaluated the effect of Hal on both enzymes activity. Simultaneously, the direct impact of Hal on the same parameters was estimated in vitro.

Materials and Methods

Animals

We tried to protect animals and used their minimum amount necessary to obtain reliable results. When choosing the number of rats in groups, we focused on similar works (Lourenço et al. 2005; Gumulec et al. 2013). The experiments were conducted on 17 outbred male rat sibs. The animals were kept in standard conditions ad libitum. In experimental group rats ($N = 10$) were daily injected intraperitoneally with 0.2 ml Hal (Gedeon Richter), 0.5 mg/kg, for three weeks. The control group ($N = 7$) received 0.2 ml saline. Rats were euthanized on the 21st day, blood serum, blood plasma and peritoneal exudate were obtained. Three rats were additionally used for experiments in vitro. The protocol was approved by local Bioethical Commission.

Determination of Hal

A LC–MS/MS method was used for analysis of the Hal concentration in rat serum as described in (Miroshnichenko and Baymeeva 2018). The analyte and internal standard (anastrozole) were extracted from serum by liquid extraction and separated on a Zorbax Bonus-RP column using acetonitrile–0.2% formic acid. Detection was performed using Agilent 6410-2 K Triple Quad LC-MS (Agilent Technologies, Palo Alto, CA, USA). The assay was linear over the concentration range 0.25–50 ng/mL with the lowest limit of 0.25 ng/mL.

Peritoneal Macrophage Activity Assay

10 ml PBS, pH 7.4 was injected intraperitoneally, abdominal cavity was opened and cell suspension was transferred into a 12-well plate with glasses at the bottom. Cells were incubated for 30 min at 37 °C. After cells binding to glass surface PBS replaced with DMEM/F12 (10% of fetal calf serum, penicillin/streptomycin) and cultivated at 37 °C in 5% CO2 atmosphere. To evaluate the direct action of Hal on cells, they were incubated for 24 h in the presence of Hal or for 2 h in presence of standard inflammation inducer PMA. The change in cell shape under the action of Hal was evaluated using a parameter «circularity» (4pi (cells area)/perimeter^2)) defined in the Image J program. The phagocytosis was measured by adding of 2 μm latex bids (Paneco) to macrophages in a ratio 10:1 and incubated them for 1 h at 37 °C. Cells were fixed with 1% gluteraldehyde and then phagocytic index (PhI, product of phagocytizing cells percent and average number of bids in cell) was evaluated.

Measurement of NO Production

NO is a short-living metabolite that is oxidized to nitrates and nitrites, therefore the concentration of these anions was determined in order to evaluate NO production. The blood serum was deproteinized by 1.5% zinc sulfate for 15 min following by centrifugation at 10000 g for 5 min. Nitrates were reduced to nitrites with cadmium for a night at room temperature. Nitrite concentration was measured colorimetrically in reaction of diazotation by sulfanilamide nitrite (Griess reaction) at wave length 540 nm (Ultrospec 1100, Amersham).

iNOS Expression

iNOS expression was estimated in macrophages by immunofluorescence microscopy (Imager M1 Carl Zeiss). Cells were fixed with 3% paraformaldehyde and permeabilized with

methanol. Then the cells were consecutively incubated with rabbit polyclonal antibodies to iNOS (Abcam, USA) and goat antirabbit antibodies, conjugated with fluorescent dye DyLight488 (Thermo Scientific). The images were analyzed in ImageJ program. Average fluorescence was estimated in 100–150 cells.

CS Activity Assay

The activity of CS in blood serum was analyzed in vitro using protozoa *Tetrahymena pyriformis*. The number of active ciliates in medium containing 0.62% test blood serum was calculated by "Biolat" analyzer (Ivanov et al. 2016). CS activity was expressed as inversely proportional to the time of death of the half the ciliates (T50). The direct toxic effect (LC50) of Hal (1–100 μM) was estimated after two hours' exposure with the drug.

Activity of the Hemostasis System

Hemostasis was evaluated on "Biolat" analyzer by the velocity of fibrin clot formation in citrated blood plasma stimulated by thromboplastin solution in 0.03 M tris-HCl, 0.025 M Ca2+, pH 7.5 (Diagem P, Russia). The formation of a blood clot (the lag phase, min) and maximum clot size (absorption units) were registered.

Leukocyte Elastase Activity Assay

Serum LE activity was analysed using spectrophotometer Ultrospec 1100 (Amercham) by the rate of specific chromogenic substrate BOC-Ala-Onp (ICN Biomedical Inc) hydrolysis and expressed in nmol/min × ml. Direct effect of Hal on LE activity was studied by adding to reaction mixture 1–100 ng/ml of antipsychotic.

Glutathione Metabolism Enzymes Activity Assay

GR activity was evaluated by NADPH oxidation in reaction of oxidized glutathione reduction. GT activity was estimated by the speed of chromogenic glutathione conjugates formation with 1-chloro-2.4-dinitrophenyl. The measurements were conducted on spectrophotometer Beckman DU-800. Direct Hal effect on enzymes was evaluated by adding Hal (1–100 ng/ml) to the reaction mixture.

Statistical Analysis

The normality was estimated by Shapiro-Wilcoxon test. One-way ANOVA and Student's test were used for comparison of parametric, while Mann-Whitney U-test was used for non-parametric data. The dispersion analysis was performed using the Fisher test. Correlations were tested using the Pearson test. Data are presented as mean ± SEM.

Results and Discussion

The subchronic Hal administration significantly changed two parameters: the activity of CS was twice decreased and the time of clot formation (lag phase) was twice increased. The analysis of variance also showed significant intergroup differences in these two parameters (F-test 3.5 and 4.6, $p < 0.05$, respectively). There were no significant differences in other parameters (Table 1).

Effect of Hal on Complement System

In addition to intergroup differences we found a negative correlation of Hal serum concentration with CS activity ($r = -0.71$, $p < 0.05$). On the surface of *Tetrahymena pyriformis* cells there are both protein that activate the classical pathway (Takahide 2018) and mucopolysaccharides that activate the lectin pathway (Wolfe 1986). In which of these pathways affects Hal needs to be further clarified. It is interesting to note that Hal effect on CS activity was individual: in 6 rats CS activity was almost twice decreased whereas in 4 rats it was at the level of control group. In vitro experiments showed that Hal in concentrations up to 100 nM was not toxic for ciliate (LC50 9 μM) and had no direct effect on the activity of CS. Apparently, the detected individual effect of Hal is determined by the individual characteristics of the cytochrome system (Murray 2006). Hal level in rat blood indeed varied significantly from 0.64 to 2.98 ng/ml. Since the majority of CS components are produced by the liver, the decrease in CS activity after sub-chronic administration of Hal can be explained by the toxic effect of the drug on hepatocytes (Bogdan et al. 2011; Kehinde 2017). This hypothesis was indirectly confirmed by the comparison of CS activity with drug concentration in samples according to mass spectrometry data. CS activity was significantly lower in the group with high Hal level (1.2 ± 0.3 ng/ml) than in the group with low Hal level (0.7 ± 0.05 ng/ml), respectively, 2.36 ± 0.67 and 3.4 ± 0.15 min, $p < 0.01$.

Effects of Hal on Hemostasis

It is known that complement and hemostasis systems are parts of the integrated serine proteases system (Markiewski et al. 2007). It is important for body protection against external invasion and internal damage. No significant correlations between CS activity and hemostasis parameters were discovered in our research. However, the analysis of the reaction of these

Table 1 The effect of Hal subchronic administration on experimental parameters

	Control	Experiment
Haloperidol, ng/ml	–	1.07 ± 0.24
CS activity, min	21.7 ± 2.1	40.9 ± 9.1 *
Phagocytic index	0.35 ± 0.02	0.29 ± 0.05
Coagulation lag phase, min	4.62 ± 0.22	8.41 ± 1.44 *
Maximum clot size, AU	11,591 ± 775	10,608 ± 786
NO, μM	5.54 ± 0.45	5.61 ± 0.32
Elastase, nmole/min*ml	4100 ± 315	3876 ± 357
Glutathione-reductase, micromole/min*ml	11.14 ± 0.77	11.12 ± 0.89
Glutathione-S-transferase, micromole/min*ml	45.31 ± 1.65	43.82 ± 0.99

*- the difference from control, $p < 0.05$

systems to the introduction of Hal indirectly showed the existence of such a connection. Indeed, a twofold decrease in CS activity coincides with a twofold increase in blood clotting time (lag phase). On the other hand the lag phase did not correlate with Hal level (unlike CS activity) and Hal had no effect on the maximum size of the clot (Table 1).

Effects of Hal on the Activity of Macrophages

PhI values did not differ significantly in the control and experimental groups (Table 1). However, a positive correlation between PhI of peritoneal macrophages and Hal level in serum ($r = 0.78$, $p < 0.05$) was found. It is known that PhI increases

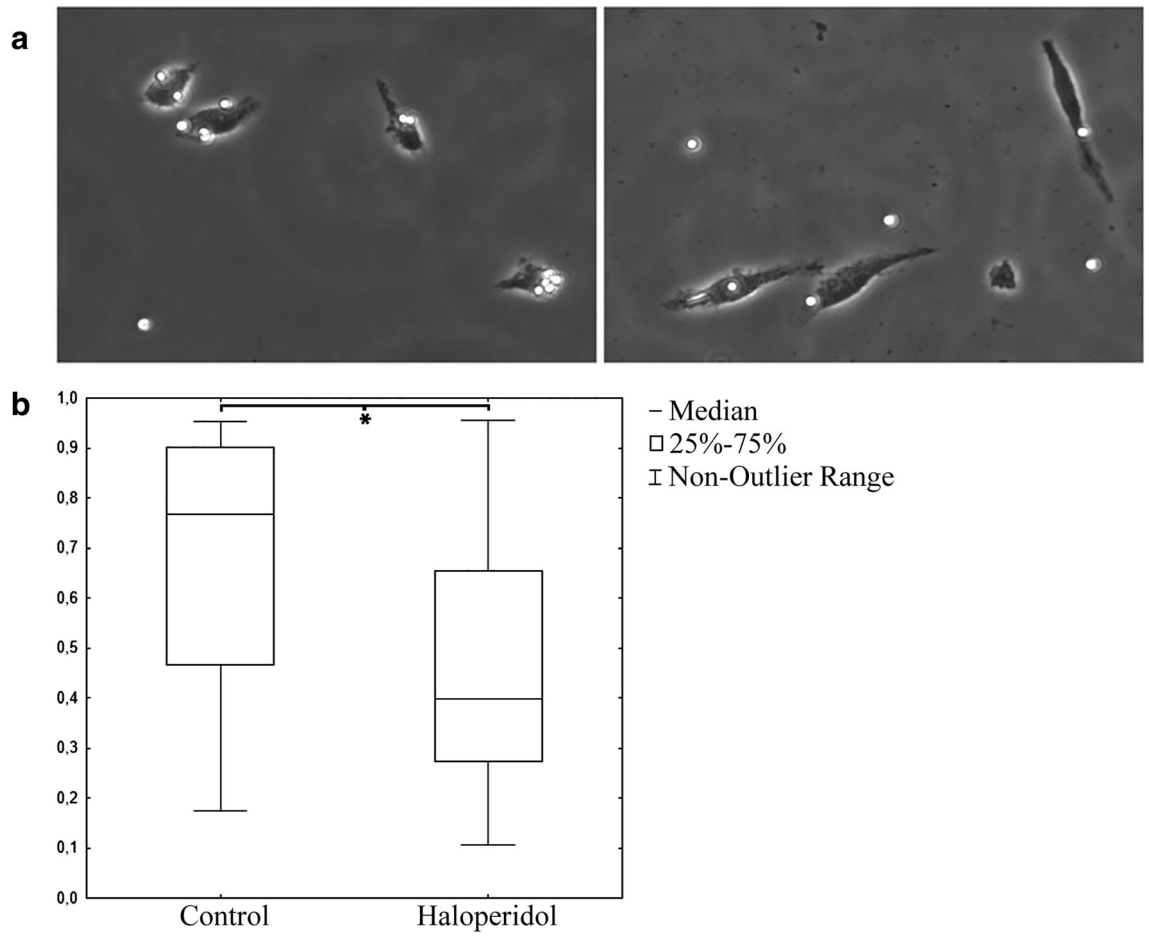

Fig. 1 **a** - Haloperidol-induced morphological changes in peritoneal macrophages (elongated cell shape). Right photo - control, left - experiment. White dots are particles of latex. **b** - Cells shape quantification under Hal treatment. Circularity calculated for 150 cells in each group are presented. * - difference between the groups, Mann-Whitney U-test, $p < 0.01$

with the transition of the functioning of resident macrophages to anti-inflammatory phenotype (Tarique et al. 2015). Thus, the detected correlation points to the" anti-inflammatory" effect of Hal. Moreover, the in vitro experiment showed that the addition of Hal (12 ng/ml) to culture fully blocks PMA-induced iNOS expression in macrophages, changes their morphology to elongated cell shape and significantly ($p < 0.01$) decrease the circularity (Fig. 1). The effect of PMA is due to its ability to activate protein kinase C. The fact that Hal blocks PMA-dependent activation indicates the interaction of Hal with the components of the signaling pathway located after this kinase. That is, the effect of Hal on macrophages is not related to its interaction with any receptors on the surface of these cells. The appearance of so-called spindle cells (65% in experimental group and only 13% in control) is also an evidence of an "anti-inflammatory" transformation (Frances Y. McWhorter et al. 2013). Interestingly, the sub-chronic intake of Hal did not cause the appearance of spindle-shaped cells. This phenomenon requires further investigation. Nevertheless, to our knowledge, this is the first evidence of Hal direct effect on the activity of macrophages in vitro.

Effects of Hal on the Leukocyte Elastase Activity

Apparently, macrophages are not the only Hal target in the immune system. Subchronic injections of Hal did not significantly change the activity of LE in the blood (Table 1). However, the negative correlation between Hal level and LE activity was revealed (−0.71, $p < 0.05$). Azurophilic neutrophil granules are the source of LE in blood and activation of these cells causes their degranulation and LE release. A negative correlation between the Hal and LE activity suggests the ability of an antipsychotic to reduce the activity of the neutrophils. In the in vitro experiment no direct effect of Hal on LE activity was revealed. It can be assumed that the action of Hal is also indirect and is possibly associated with its toxic effects on hepatocytes, which are the main source of LE inhibitor alpha1-antitrypsin.

Table 2 The influence of Hal on the correlation of the studied parameters

	Control	Experiment
NO vs Elastase	*ns*	- 0.68, $p < 0.05$
NO vs Reductase	*ns*	0.75, $p < 0.05$
NO vs Transferase	- 0.71, $p < 0.05$	−0.68, $p < 0.05$
NO vs Hemostasis lag phase	- 0.81, $p < 0.01$	−0.78, $p < 0.01$
Elastase vs Reductase	*ns*	- 0.82, $p < 0.05$
Elastase vs Phagocytic index	*ns*	- 0.70, $p < 0.05$

ns - not significantly

Effects of Hal on Antioxidant Enzymes Activity

The subchronic Hal injections did not change the activities of both antioxidant enzymes (Table 1). The in vitro experiment also did not reveal direct Hal effect on GT and GR activity. However, a negative correlation between Hal serum concentration and GT activity ($r = -0.67$, $p < 0.05$) was found. It is known that long-term administration of typical and atypical neuroleptics increases oxidative stress and cell damage and affects the level of expression of antioxidant enzymes (Schmidt et al. 2009). The direct cytotoxic effect of neuroleptics, in particular, Hal and clozapine, was also described (Polydoro et al. 2004; Kropp et al. 2005). The correlations we have identified can be explained by these mechanisms.

Effects of Hal on General Homeostasis

At the final stage of the study we conducted a comparative analysis of the correlations between all parameters in the experimental and control groups (Table 2). The correlation between the NO concentration and the activity of the GT, as well as between the NO concentration and the lag phase of coagulation was observed in both groups and were similar. It is interesting to note that under the influence of sub-chronic effects of antipsychotic appeared a number of new correlations. The significant correlations between NO and LE and GR activity, LE activity and activity of GR, LE and PhI of peritoneal macrophages were found. It can be assumed that sub-chronic administration of Hal significantly affects homeostasis and causes a number of complex transformations, the mechanism of which requires further investigations.

Conclusion

This pilot study despite the presence of certain limitations at small sample sizes, for the first time showed, that subchronic administration of Hal significantly reduces the functional activity of CS and inhibits hemostasis. The comparison of in vivo and in vitro data showed that Hal has a direct effect on phagocytic component of innate immunity and an indirect effect on LE and antioxidant enzymes. We believe that the indirect effect is due to the toxic effect of Hal, primarily on hepatocytes. Our research confirms that during the subchronic antipsychotic administration it is necessary to prescribe drugs which normalize the state of CS and antioxidant defence and to constantly monitor blood coagulation system. The biological test system based on the use of eukaryotes *Tetrahymena pyriformis* provides ample opportunities to monitor the activity of CS in the blood of patients, as well as to assess the toxicity of psychotropic drugs.

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