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Effects of haloperidol on striatal neurons: relation to neuronal loss (a stereological study)

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Abstract

In the present work, we investigated the effect of chronic haloperidol administration on the number of striatal neurons in guinea pigs. For this purpose, adult male guinea pigs were given daily injections of 1, 2 or 3 mg/kg of haloperidol for 6 weeks. After treatment, the animals were anesthetized via brief inhalation of ether, the brains were removed and the corpus striatum was dissected. Then the tissues were processed and semi-thin sections were stained with toluidine blue for stereological and histopathological evaluation. The physical disector was used for measurements of nuclear height and numerical density of striatal neurons and also to evaluate both normal and degenerated neurons within the corpus striatum of treated animals and untreated controls. In the control group, the mean numerical density of neurons was calculated as 47.92 cell/mm^3 and the mean nuclear height as 3.58 µm. Mean densities of all (both viable and degenerated) neurons were calculated to be 45.46 in the low-dose ($p < 0.01$), 39.73 in the medium-dose ($p < 0.001$) and 30.31 cell/mm^3 in the high-dose group ($p < 0.001$). Mean densities of degenerated neurons in the low, medium and high dose group were 30.72, 22.93 ($p < 0.001$) and 15.56 cell/mm^3 ($p < 0.001$) respectively. Mean nuclear heights were 2.804 ($p < 0.0001$), 2.78 ($p < 0.0001$) and 2.33 µm ($p < 0.00001$) in the low, medium and high dose group, respectively.

These results suggest that haloperidol treatment may lead to loss of neurons and decrease in the nuclear size within the corpus striatum.

Key words: *haloperidol, striatum, stereology, physical disector, Guinea pig, histology.*

Introduction

Antipsychotic drugs (APD) are widely used in the treatment of schizophrenia as well as other psychotic disorders. Of these APDs, the traditional/typical antipsychotic drugs, such as haloperidol, are still frequently prescribed despite the acute and delayed extrapyramidal side effects including pseudoparkinsonism, acute dystonia and tardive dyskinesia that are frequently associated with their use [1-3].

Haloperidol was developed in the late 1950s for use in the field of analgesia. Research subsequently demonstrated effects on hallucinations, delusions, aggressiveness, impulsiveness and states of excite-

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ment and led to the introduction of haloperidol as an antipsychotic. Haloperidol is frequently prescribed for the treatment of positive symptoms of schizophrenia. The drug, a butyrophenone, acts principally as a D-2 dopamine receptor antagonist but also shows a high affinity for sigma binding sites [4]. Haloperidol, a commonly prescribed typical neuroleptic, is known to be toxic *in vitro*, possibly as a consequence of its conversion to pyridinium-based metabolites and potentially by raising glutamate-mediated transmission. This condition, which is associated with prolonged treatment with neuroleptics, typically involves involuntary movements in the orofacial region but may also include choreic movements of the trunk or extremities [5].

Over the recent years design-based stereology has become the state-of-the-art methodology in quantitative histological analyses, and the application of design-based stereological methods to the analysis of the CNS has considerably contributed to our understanding of the functional and pathological morphology of many biological structures [6-8].

The physical disector was developed as an unbiased and efficient stereological method to estimate cell number in a region. This tool gives a reliable estimation of particle number and size in an anatomically defined area [9]. Therefore, the quantitative analyses of neurons may become a very significant finding in these kinds of studies when assessing cell proliferation, dimensional changes or degeneration following different drug cures.

There were tested in our study some common hypotheses about potential effects of haloperidol treatment in the following aspects: i) effects of chronic haloperidol injection at different doses, ii) detection of damage level with quantitative data in terms of both numerical density and nuclear height via the physical disector, and iii) examination of histopathological and structural changes on the same sections.

Material and methods

Experimental design and drug application

In the present study, 20 adult male guinea pigs (Ataturk University Experimental Research and Applying Center) were kept on a 12-h light: 12 h dark cycle with food and tap water available ad libitum. Each treatment group consisted of 5 guinea pigs that received daily intra-peritoneal injections (once a day at 9 a.m.) of either saline or haloperidol in different doses for 6 weeks according to the following schedule: I) haloperidol 1 mg/kg, *i.p.* (low-dose group); II) haloperidol 2 mg/kg, *i.p.* (medium-dose group); III) haloperidol 3 mg/kg, *i.p.* (high-dose group); IV) saline vehicle, *i.p.* (control group). All experimental protocols were approved by the Ethical Committee at the Atatürk University.

Drugs and chemicals

Haldol® (Decanoate 100 mg) was obtained from Ortho-McNeil Pharmaceutical (San Bruno, CA; USA).

Perfusion and fixation

Following the drug-treatment, all animals were anesthetized via short inhalation of ether, and then perfused intracardially using a 0.9% saline (30 ml) solution, followed by a mixture of 2% paraformaldehyde + 2% glutaraldehyde (150 ml) in 0.1 M phosphate buffer, pH 7.4 for approximately 30 minutes, at room temperature. Brains were removed and stored in the same fixative overnight at 4°C [10].

Histological procedure

On the following day, the striatum in each brain was dissected out as described by Turner and Shetty [11] and the striatal tissue samples were post-fixed in 1% osmium tetroxide for 1 h, dehydrated through a graded acetone series, and embedded in araldite CY 212. Each araldite-embedded sample was cut into serial sections with an LKB Nova Ultratome (Bromma, Sweden) and the slides were stained with toluidine blue.

Stereological method

The selection of the physical disector pairs was done as described by Sterio [12]. We obtained approximately 80 sections from each striatum.

Based on the pilot study, pairs from every 4th section were chosen randomly, and in this way approximately 15-20 section pairs were obtained. This number is in an acceptable range for stereological analysis [13-16]. Disector pairs were taken from the tissue at a known interval, until the tissue sample was exhausted. Two consecutive sections were mounted on each slide. Photographs of adjacent sections were taken with a digital camera (Olympus BH-2, Japan) at a magnification of ×400. Nucleoli of neurons seen in

the reference section but not in the look-up section were counted. To increase the countable particle number, i.e. nucleoli, we exchanged the role of sections in the second step. An unbiased counting frame was placed on the reference and the look-up sections on the screen of the PC to perform the counting according to the disector counting method. The bottom and the left hand edges of the counting frame are considered to be the exclusion lines together with the extension lines. Other boundaries of the frame and the top-right corner were considered to be inclusion points and any particle that hit these lines or was located inside the frame was counted as a disector particle [17-19] (Fig. 1).

The mean numerical density of neurons ($N_{V(neuron)}$) in neurons per mm^3 was estimated using the following formula [9,20].

$$N_{\upsilon(neuron)} = \sum Q^{-}(neu)/t \times A$$

Where $\Sigma Q^{-}_{(neu)}$ is the total number of nucleoli counted in the reference section; t is the mean section thickness (1 µm), and A is the area of the unbiased counting frame.

Finally, histopathological examinations were carried out on images of the same sections.

Statistical analysis

To evaluate the significance of observed differences, we used the Student's t test (two tailed, significance limit is $p = 0.05$ in this test). All statistical calculations were performed using SPSS 13.0 for Windows.

Results

Stereological results

In this study, the mean numerical density and mean nuclear height were estimated for all neurons, including viable and degenerated neurons in striata of haloperidol-treated groups and compared to the control group.

We observed that the neuronal densities of the experimental groups were significantly decreased in comparison to the control subjects (Fig. 2A). The mean neuronal densities in the low-dose ($p > 0.05$), medium-dose ($p < 0.001$) and high-dose ($p < 0.001$) groups were reduced by 5.14%, 17.09%, 36.75% respectively, relative to the control group. We have found statistically significant differences when comparing the mean neuronal density of experimental groups with each other (low-dose group vs. medium-dose group $p < 0.0001$ and medium-dose group vs. high-dose group = $p < 0.0001$).

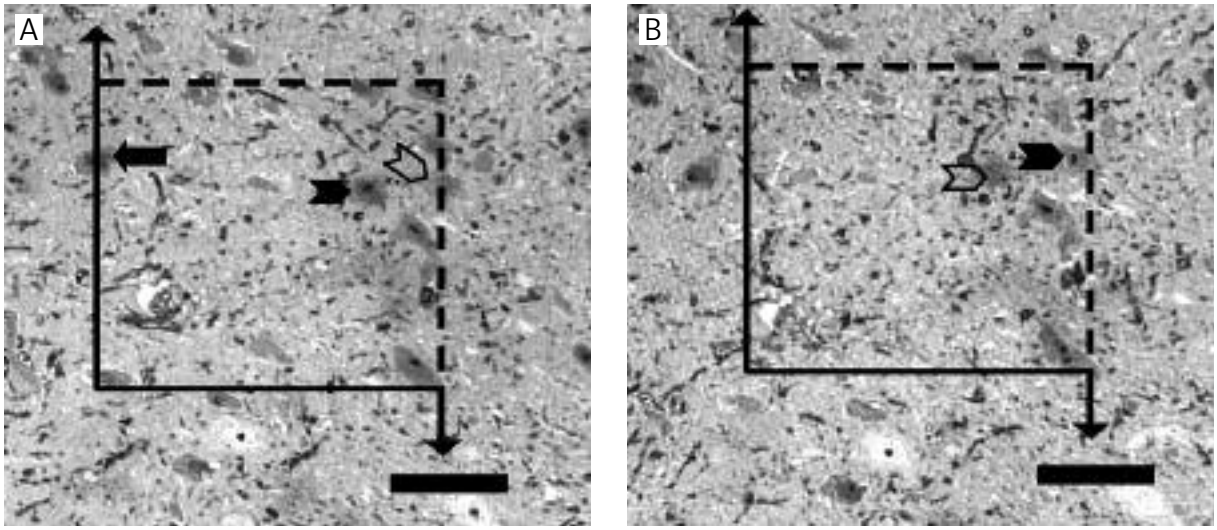

Fig. 1. An application of the physical disector counting method. The same areas of two adjacent sections, separated by 1 µm, are shown **A. B**) Nucleoli hitting the exclusion lines were excluded (arrow) from counting. Nucleoli hitting the inclusion lines or located inside the frame (black arrow head) were counted as disector particles if their profiles (transparent arrow head) are not seen in the look-up section B. Magnification Bars = 25 µm.

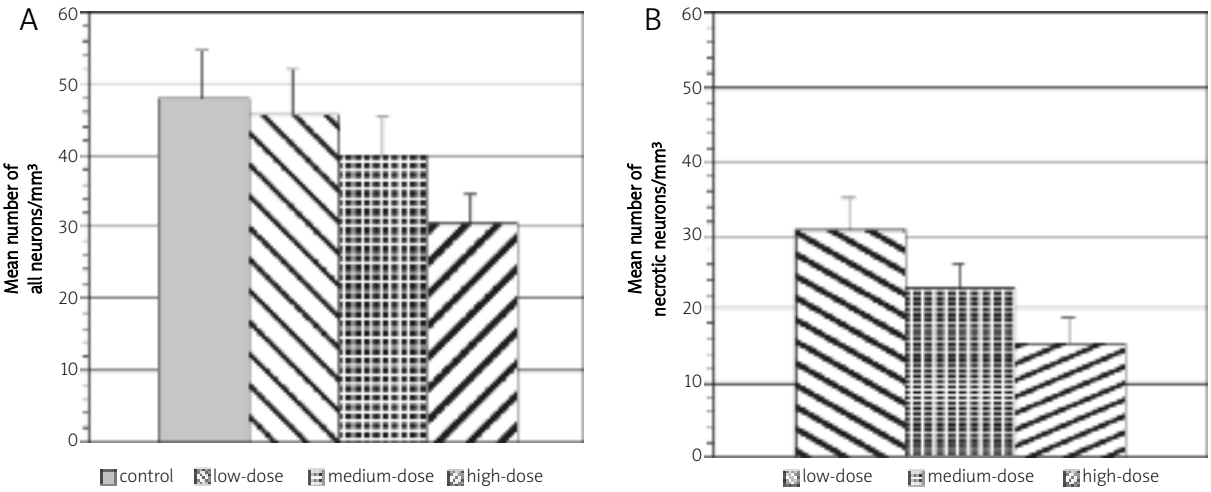

Fig. 2. A) Histogram showing the mean numerical density of all neurons in the striatum of guinea pigs, treated with haloperidol 1, 2 and 3 mg/kg (once a day) for six weeks and controls ± SEM. A significant reduction in the percentage of neuronal density was found with haloperidol, especially at the dose of 2 mg/kg and 3 mg/kg, compared to control. **B**) The mean numerical density of degenerated neurons in the striatum of the same pigs. There was a significant dose-dependent decrease in the numerical density of degenerated neurons within the striatum among treatment groups.

We also found that the density of degenerated neurons gradually decreased from low-dose to high-dose group (Fig. 2B). Mean percentages of the degenerated neurons were 67.57%, 57.71% and 51.33% in the low-, medium- and high-dose group, respectively. There was also a statistical difference in terms of density of degenerated neurons among treatment groups (low- and medium-dose groups; $p < 0.001$ medium- and high dose groups; $p < 0.001$).

Histological results

A structural analysis of the striatal neurons was made under the light microscope (Fig. 3). There was evidence of chromatin condensation and cytoplasmic shrinkage in the striatal neurons of haloperidol-treated animals, suggesting necrosis. The mean numerical density of striatal neurons (including degenerated ones) decreased significantly in the high-dose group relative to the low-dose group, suggesting lysis or ingestion of the degenerated cells.

Discussion

Firstly this study was limited to a 42-day period. In rats, the chronic treatment period during 42 days corresponds to about 6 years of treatment in patients [21]. Secondly we investigated the effects of different doses of haloperidol on the numerical density of neurons in the striatum by using the physical disector counting method [9,10,22].

The cholinergic system has been implicated in the pathophysiology of schizophrenia [23,24]. Although the mechanism of haloperidol-induced cell degeneration is not well understood, our previous report indicated that one of the possible effects causing the damage is vasoconstrictor effects of haloperidol administration [22]. Investigations have shown a decrease in the number of neurons of the striatum in the brains of individuals suffering from schizophrenia [25]. Yet, no evidence of a decrease in density of neurons was found [25-27]. However, no unbiased stereological investigation was used in these studies.

Morphometric results of this study were fairly interesting, especially when examined all together. These results are discussed below:

1) Morphological studies have suggested that neuroleptics act as neuroprotective agents by stimulating neurogenesis [21]. Eggerman and Zahm declared that the numbers of some neuronal bodies in striatal and ventral striatal structures of haloperidol-treated rats greatly exceeded those observed in the same structures of control animals [29]. Moreover, Merchant *et al*. indicated that low-dose haloperidol might be beneficial and the number of

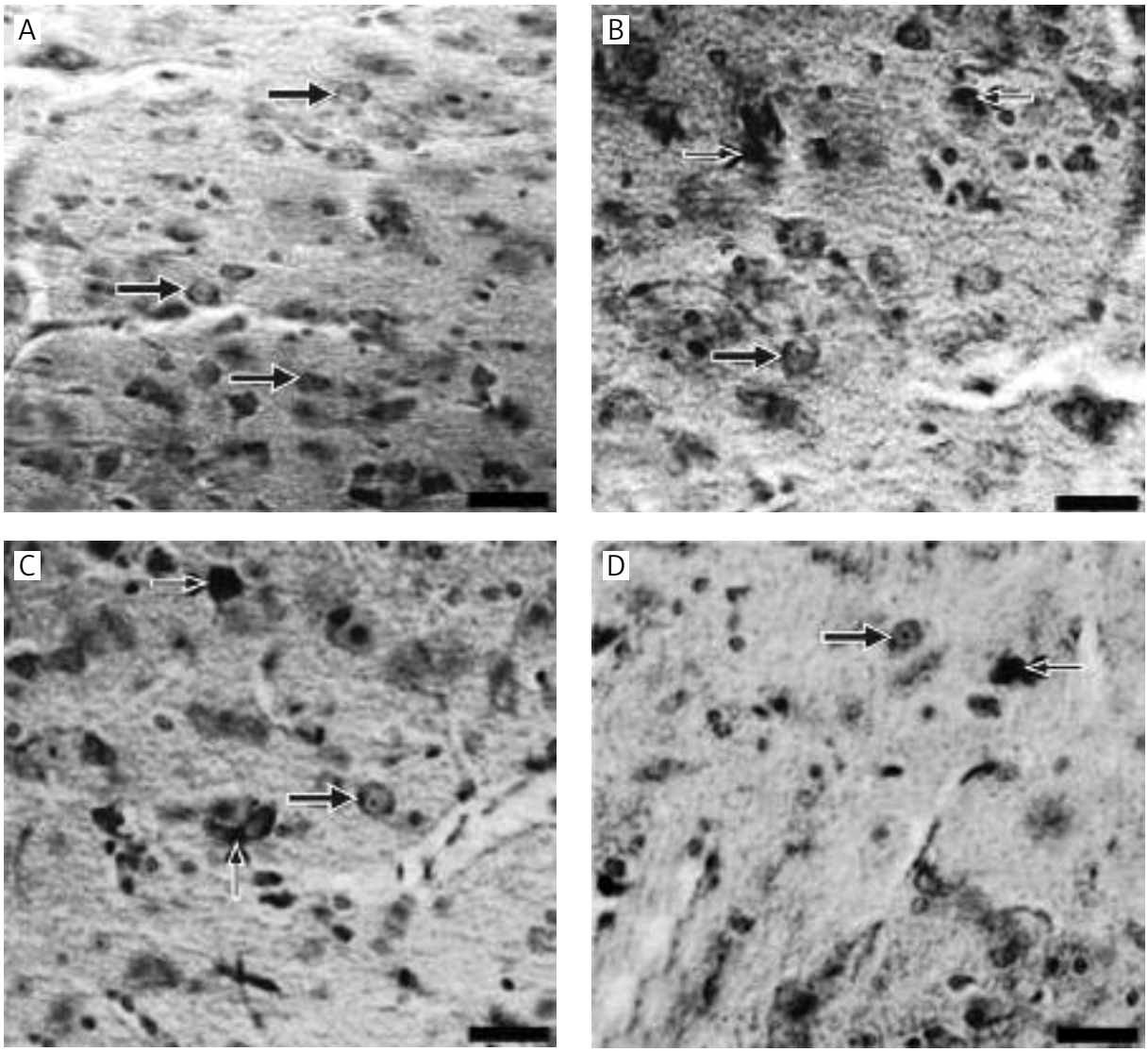

Fig. 3. Light micrographs of the striatum of guinea pigs. **A-D** show view of control, low-, medium- and high-dose group, respectively. Thick arrow shows healthy neuron, thin arrows indicate degenerated neurons. Magnification Bars: 20 μm.

cells did not appear to be affected by this treatment [30].

We certainly determined that the numerical density of striatal neurons was decreased after haloperidol treatment. When the low (5.14%, $p > 0.05$), medium (17.09%, $p < 0.001$) and high (36.75%, $p < 0.001$) dose haloperidol-treated groups were examined by the physical disector counting method, it was detected that the numerical density of striatal neurons was significantly low in comparison to that of the control group. Also results of the treated groups were different from each other (low-dose group vs. medium-dose group, $p < 0.0001$; medium-dose group vs. high-dose group, $p < 0.0001$).

Some studies have reported like our data that treatment with haloperidol may have side effects on neurons [31,32]. It has also been reported that treatment with these agents may have side effects, such as neurodegeneration or death of neurons in the hippocampus [10,33], striatum [34] and medial prefrontal cortex [35]. Mitchell and co-workers have suggested that chronic administration of haloperidol could induce cumulative neuronal loss in the

substantia nigra pars reticulata and thereby induce the pathological changes [36].

When estimating numerical density of all neurons, it was clearly observed that neuronal loss occurred especially in the high-dose group. If we considered only these data, we could not distinguish an effect of a low dose and we might suggest that low-dose haloperidol treatment does not have any side effect on the brain, because there was no statistical difference in numerical density between the control and low-dose groups.

2) It was found that the densities of degenerated neurons in the experimental groups were gradually decreased from the low-dose to the high-dose group; in order of the low-, medium- and high-dose group the values were 67.57%, 57.71% and 51.33%. There was also a statistical difference in terms of density of degenerated neurons both between the low- and medium-dose groups ($p < 0.001$) and medium- and high-dose groups ($p < 0.001$). When nuclear height of degenerated neurons was evaluated in all groups, mean nuclear height reductions were 35.48% ($p < 0.0001$), 39.95% ($p < 0.0001$) and 46.93% ($p < 0.00001$), respectively, compared with that of the neurons in controls. There was also a statistical difference in height of the degenerated neurons between both the low- and medium-dose groups ($p < 0.05$) and medium- and high-dose groups ($p < 0.05$). When evaluating numerical density of degenerated neurons, numerical density of the high-dose group was less than that of the other treated groups; thus degenerated cells would die by shrinking and then these cells would disappear.

3) The pathway of haloperidol-induced cell death is still discussed today. Some researchers have reported that haloperidol increases p53 expression, leading to apoptosis [32]. A preliminary study was accompanied by an increase in the number of apoptotic cells in the striatum [36]. Other studies have reported haloperidol and reduced haloperidol induced cell death via apoptosis [37]. A cell loss in the treated groups could be due to excitotoxic cell death, since long-term haloperidol administration has been reported to increase striatal glutamatergic activity [34,38]. Previous studies have shown that excitotoxic mechanisms may be involved in the development of neuroleptic-induced neurodegeneration in rat [34]. Oxidative stress could also play a role in the toxic response. Haloperidol induces free radicals in vitro [3] and clinical studies have shown increased markers of oxidative stress in schizophrenic patients [39] and beneficial effects of antioxidative treatment [32].

We thought in this subject that all possible mechanisms mentioned above were triggered by arterial vasoconstriction as indicated in our previous paper [22,40]. Present histological data show that haloperidol causes neurodegeneration via necrosis in the striatum like our previous report [10]. According to current histological findings, there was evidence of chromatin condensation and cytoplasmic shrinkage, suggesting necrosis. In terms of haloperidol dosage, the number of degenerated neurons decreased significantly in the high-dose group relative to the low-dose group.

In conclusion, haloperidol may have been toxic side effects on the neurons, and it is possible that a cognitive impairment might be expected as a result of striatal neuron loss after not only high-dose but also low-dose chronic haloperidol treatment. Consequently, in clinical settings, neuroleptic treatment with haloperidol should be avoided, even in a lower dose.

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