

RESEARCH ARTICLE

Beneficial effects of metformin on haloperidol-induced motor deficits in rats. A behavioral assessment

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Objective: One of the most common side effects of haloperidol is the extrapyramidal syndrome, resulting from inhibition of nigrostriatal dopaminergic circuits and mitochondrial dysfunction due to structural similarities to pyridinium derivative, MPP⁺ that induce oxidative stress. In exchange, the use of metformin appears to enhance neurogenesis, energy metabolism, and oxidative status, so these properties can be speculated in the context of drug-induced pseudoparkinsonism by haloperidol. **Methods:** To assess motor coordination and activity, rodents were divided into four groups: CTR (n = 10) - animals that received distilled water, METF (n = 10) - animals that received metformin 500 mg / kgbw, HAL (n = 10) - animals that received haloperidol 2mg / kgbw, HALMETF (n = 10) - haloperidol 2mg / kgbw and metformin 500 mg / kgbw. The treatment was administered for 34 days at the same time by gastric gavage, during which time behavioral tests, rotarod (days 7, 14, 21, 28), catalepsy (day 30), open field (day 32) and novel object recognition (day 34) were performed. **Results:** The monitored parameters, showed significant differences between the groups of interest (HAL and HALMETF respectively), so that the administration of metformin at the beginning of treatment reduces the cataleptic behavior. The HALMETF group shows an attenuation of the motor deficit during the rotarod test and the freezing period from the Open Field test, is diminished. **Conclusions:** Metformin treatment has a beneficial effect in haloperidol-treated rats, demonstrated by decreased cataleptic behavior, improved motor performance and reduced haloperidol-induced anxiety behavior.

Keywords: metformin, haloperidol, rat, behavior, coordination

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Introduction

Contrary to the fact that progresses have been made in the medical field, due to the complex physiology of the brain, for many of the mental disorders, pharmacological approaches could not meet the therapeutic needs [1]. In the case of schizophrenia but also in other neurotic disorders, despite the development of modern neuroleptics, one of the most widely used antipsychotics remains haloperidol given its low costs and high potency [2]. From a pharmacotherapeutic view, haloperidol exerts its effects on the central nervous system (CNS) through dopaminergic antagonistic action with competitive blocking of postsynaptic D₂ dopaminergic receptors in the mesolimbic system [3]. Extrapyramidal effects, mainly pseudoparkinsonian symptoms are due to blockade of the same receptor subtype in the nigrostriatal dopaminergic circuit [4] and are often irreversible. In addition to this mechanism, the blockade of complex I (NADH : ubiquinone oxidoreductase) of the electron transport chain (ETC) could lead to the same extrapyramidal symptoms by the degeneration of dopaminergic neuronal mass. Structural similarity ex-

ists between haloperidol and the pyridinium derivative of 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) [5], 1-methyl-4-phenylpyridinium (MPP⁺) that causes mitochondrial dysfunction, and a considerable amount of evidence was already published that haloperidol induces oxidative stress [6, 7]. Considering this structural similarity it was assumed that the ability of HPP⁺ to penetrate the neuron is related to a specific transporter, hOCT3 [8, 9]. Based on its property of interacting with neurotoxins and positively charged molecules, it was suggested that hOCT3 is an effective transporter of HPP⁺. Also, it contributes to the exacerbation of the irreversible extrapyramidal effects of this neurotoxic metabolite. Thus, dopamine depletion or blockade of dopaminergic receptors causes an imbalance of monoaminergic metabolism that further leads to defects, especially motor impairment.

Considering the fact that research on human subjects is restricted, most of the data on behavioral and neurochemical effects come from animal models. Many of these studies present data obtained after acute treatment, but the therapeutic efficacy occurs over time, which implies that a more appropriate animal model is that obtained after chronic treatment [10].

Drug-repurposing, a promising strategy to accelerate the drug discovery process, has already been suggested for met-

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formin [11, 12]. In addition, rodent studies indicate that chronic use of metformin prevents the adverse effects of aging and increases life expectancy [13, 14], and data from a meta-analysis confirms decreased risk of Parkinson's disease in combination with sulfonylurea derivatives [15]. As it was previously demonstrated, the effects of metformin occur are attributed to the AMP-activated protein kinase (AMPK) activation and phosphorylation followed by increased peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α) expression [1, 16] of muscle coordination and activity [17]. *In vitro* models show that metformin is able to stop the apoptotic process via AMPK pathway [18], being a possible therapeutic target in neurodegenerative diseases [7]. However, the effects of metformin on the CNS of experimental animals are poorly studied. Even if metformin is able to block the complex I of the electron transport chain, the doses of metformin used do not produce reactive species [1, 19].

Therefore, the aim of this study, was to investigate the influence of metformin on the behavioral changes induced by haloperidol, and its ability to prevent or delay the onset of neuroleptic-induced parkinsonian symptoms in rats.

Material and Methods

The study was conducted in compliance with all experimental procedures in accordance with European Directive 2010/63 / EU and was approved by the Ethics Committee for Scientific Research of the George Emil Palade University of Medicine, Pharmacy, Science and Technology of Târgu Mureş (approval no. 533/2019), and by National Sanitary Veterinary and Food Safety Authority (approval no. 42/2020).

Animals and Treatment

Haloperidol (Haloperidol Richter 2 mg / ml, Gedeon Richter, Târgu Mureş, Romania) and metformin (Glucophage 500 mg, Merck Santé, Semoy, France) were purchased from the Romanian pharmaceutical market. The protective coating of the tablets was removed and tablets were powdered. The powder was then added to distilled water which served as a solvent, resulting an extemporae suspension. The experiment was performed on a number of 40 male Wistar rats aged 6 months and an average weight (\pm SEM) of 453.3 ± 2.1 grams, from the Animal Facility of

the George Emil Palade University of Medicine, Pharmacy, Science and Technology of Târgu Mureş. Upon arrival in the laboratory, the animals were placed in individual cages, with a habituation period of 7 days, handled daily for stress reduction, while the environmental conditions were standard (12/ 12 hours light-dark cycle), ambient temperature 20 ± 2 ° C, $60\% \pm 10\%$ humidity. The access to food, which consists of standard pellets for rodent, was unaltered throughout the study. Body weight was recorded once a week for dose adjustment.

Rodents were randomly divided into 4 groups: CTR (Control, distilled water, n = 10), HAL (haloperidol 2 mg / kg, n = 10), METF (metformin 500 mg / kg, n = 10), HALMETF (haloperidol 2 mg / kg and metformin 500 mg / kg, n = 10). The medication was administered for a period of 34 days, daily, between 8:00 AM and 10:00 AM with the help of an oral feeding cannula, in a volume of 1 ml / kg, in a separate room. Administration dosages, administration route and treatment duration were established based on literature findings [20, 21, 22]. Also, the sample size per each group was chosen by consulting other similar experiments on rats from the literature [23, 24].

At the end of the experiment, all animals were decapitated under anesthesia with ketamine / xylazine in a dose mixture of ketamine (100 mg / kg) and xylazine (10 mg / kg), in order to collect samples of neuronal tissue and blood for further investigations.

Behavioral assessment

The behavioral tests were performed 6 hours after the administration of the medication (except for the catalepsy test), between 14: 00-20: 00, in order to reduce the possible confounding effects of sedation and catalepsy manifested immediately after the administration of haloperidol. Study timeline of the chronic experiment and the timing of behavioral assays is shown in Figure 1. The investigators were not blinded to animal group during experiments, but the offline behavioral analysis was performed by two experienced researchers to assess inter-observer reliability and to reduce bias. During the experiment we did not consider the measurement of glycaemic levels because the stress induced in order to determine this parameter might affect experimental animal behavior and based on the fact that metformin does not influence glycaemic levels this deter-

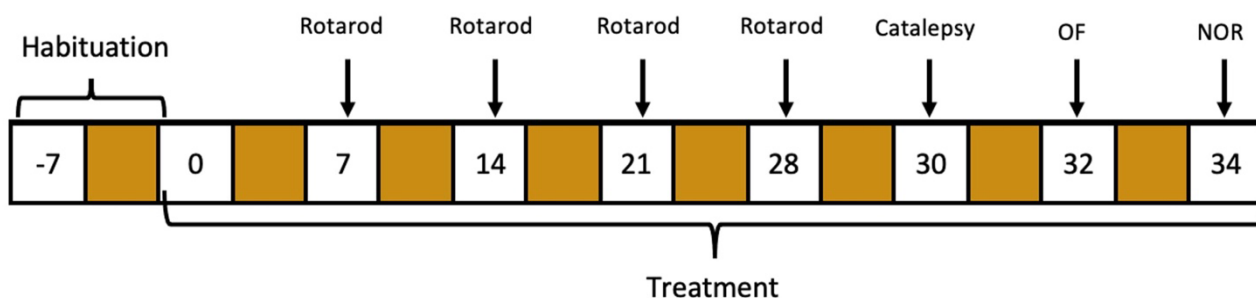


Fig. 1 Study timeline of chronic experiment and the timing of behavioral assays. The timeline shows the treatment period of 34 days preceded by a regular period of 7 days (OF – Open Field, NOR – Novel Object Recognition). Behavioral tests were performed between 14:00 and 20:00 PM.

mination was not deemed justified. Moreover the authors did not consider this parameter to be involved in the protective effect.

Catalepsy

The cataleptic effect in rats was assessed by performing the bar test method on day 30 of the experiment. The behavior of the rats was evaluated 30 minutes after medication and then at one-hour intervals, up to 180 minutes. The height of the bar was set at 10 cm and the rats were carefully positioned, with the front limbs on the bar and the hind limbs on the floor of the apparatus. The end point of the catalepsy test was considered to be the moment when both forelegs were removed from the bar or the situation in which the animal moved its head, noting the desire to explore. If the rodents failed to maintain on the bar from 3 attempts, it was considered that there is no catalepsy phenomenon. The cut-off time was considered 300 seconds. After each trial the animals were placed back in the individual cage [25].

Locomotor performance and coordination

The motor coordination was assessed on a rotarod apparatus (Ugo Basile 7700, Italy) and this test is one of the most widely used for assessing the motor coordination ability of rats. The speed was constant during the analysis, in order to obtain a higher sensitivity [26], and the endpoint of this test was considered as the moment when the animal falls from the device. The device is attached to a drum with a diameter of 5 cm that rotates depending on the speed applied by the motor. The apparatus is divided into 4 equal compartments by separating plates.

Subjects were placed in the experimental room at least one hour before the experiment in order to become accustomed to the environment. For training, the rats were placed on the rotarod at a speed of 15 rpm for 180 seconds [8], 5 consecutive days, before starting the treatment, in order to reach a stable performance. During treatment, rodents were tested weekly at a speed of 15 rpm and latency was measured until falling or for maximum of 300 seconds. After each training session and test, the device was cleaned with 70% alcohol; the temperature at which the workouts and tests were performed (once a week for 4 weeks) was maintained at $20 \pm 2^\circ \text{C}$ in a quiet room.

Open Field

The Open Field (OF) test was performed in a plexiglass box of 60 x 60 cm with black base and transparent walls, with a height of 50 cm. During the test, the animals were placed in the center of the arena. The motor and behavioral activity were recorded using a top view camera at 30 fps, for 5 minutes [27]. The arena was disinfected after each test with 70% alcohol. All trials were analyzed with EthoVision XT (Noldus IT, Wageningen, The Netherlands, version 11.5), by monitoring the moving distance of the rats and their immobilization time.

Novel object recognition

Novel object recognition test (NOR) was used to evaluate memory. The objects used were about the same height and were heavy enough so that the rodents were unable to move them from their original position. They were placed at an equal distance of 30 cm from the corners, diametrically opposite and after each analysis were wiped with 70% alcohol to limit the appearance of any olfactory bias.

Initially, two identical objects were placed for each rat for a period of 5 minutes. At the end of this period, they were returned to the personal cage, at which point the box and objects were cleaned. Subsequently, after 24 hours, the procedure was repeated, but one of the familiar objects was replaced with a new one in order to evaluate the retention process. This test lasted for 5 minutes. Exploratory activity was considered valid when rodents sniff objects or touch them with their front paws but without leaning on or sitting on them. The count of the exploration times was performed with the help of the recording software. In this context, the discrimination index (DI) represents the difference of exploration time of the new object (EB) compared to the familiar object (EA) compared to the total time spent exploring the two objects in the retention process, $DI = (EB - EA) / (EA + EB)$ [28, 29, 30].

Statistical analysis

Statistical analysis was performed using Graphpad Prism software (San Diego, California USA, ver. 8). The Kolmogorov-Smirnov test was performed to test the normality of the data and values were expressed as mean \pm SEM. For the statistical analysis of catalepsy and rotarod test results, a two-way ANOVA followed by Tukey's post-hoc test was performed to evaluate the influence of the treatment. For statistical analysis of the OF and NOR tests, one-way ANOVA test was performed. The significance level was set at p value less than 0.05.

Results

Catalepsy

All groups were subjected to the same test, but the CTR and METF groups did not show cataleptic states. There was a significant effect of the treatment ($F_{3, 144}=302.7$, $p<0.0001$), time ($F_{3, 144}=43.01$, $p<0.0001$) and a significant treatment x time interaction ($F_{9, 144}=13.91$, $p<0.0001$). Regarding the latency differences between HAL and HAL-METF, significant differences were observed regardless the time of testing. Figure 2A presents the latency to step down in the catalepsy test for rats of the METF, HAL, HAL-METF and CTR groups, respectively. An overall increase in the latency to step-down from the bar at 30, 60, 120, and 180 min after the administration was observed in HAL and HALMETF groups in relation to the CTR group.

HAL group showed an increasing tendency with time, a higher degree of catalepsy being observed at 120 and 180 minutes after administration (Figure 2B). On the other

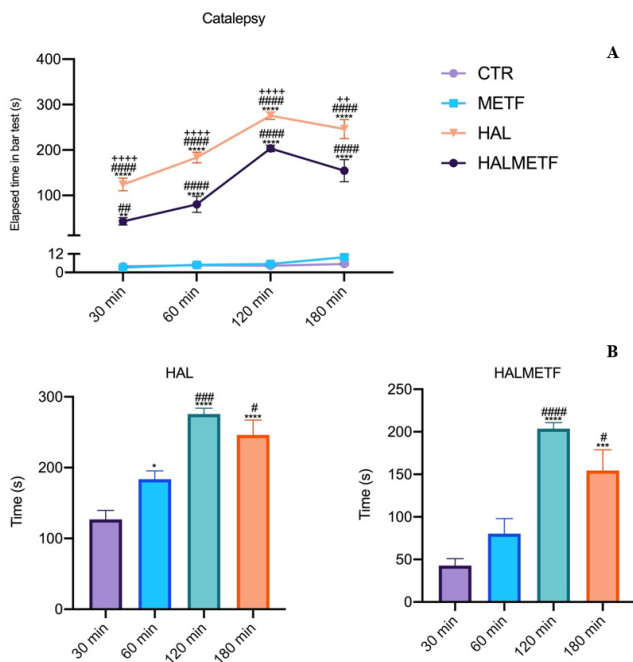


Fig. 2. (A) Latency to step down in the catalepsy test for rats expressed as seconds. Values displayed are means ± SEM. Statistically significant differences compared to the CTR group (Control group) are noted with ** $p < 0.01$, **** $p < 0.0001$. Statistically significant differences compared to the METF group (Metformin group) are noted with ## $p < 0.01$, #### $p < 0.001$. Statistically significant differences compared to the HALMETF group (Haloperidol + Metformin group) are noted with ++ $p < 0.01$, +++ $p < 0.0001$. **(B)** Differences of latency to step down in the catalepsy test for group HAL (Haloperidol group) and HALMETF. Results are expressed as mean ± SEM. Significant differences between treatment intervals are noted with * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$, compared to 30 min. Significant differences between treatment intervals are noted with # $p < 0.05$, ### $p < 0.001$, #### $p < 0.0001$ compared to 60 min.

hand, there is a similar trend of decrease of the latency to step down of the bar in the case of rats from the HALMETF group (Figure 2B), the time of maintaining the cataleptic state being shorter than in the HAL group.

Rotarod

To determine the effect of metformin on the ability to move, we tested the motor performance using the rotarod test. Rats were tested weekly by quantifying the time spent on the rod. The performance of the animals expressed as latency to fall off the rotating drum is shown in Figure 3A. A progressive decline in the case of HAL and HALMETF groups was observed. Two-way repeated measures ANOVA indicated a significant effect of treatment ($F_{3, 144} = 505.8, p < 0.0001$), time ($F_{3, 144} = 33.23, p < 0.0001$) and a significant treatment x time interaction ($F_{9, 144} = 23.22, p < 0.0001$). The Tukey post hoc test showed an overall decrease in the performance and motor coordination from the first to the fourth week of treatment (Figure 3A).

Within the HAL group, a sudden decrease in motor performance was observed in the second week, maintaining this trend in weeks 3 and 4. At the same time, differences were found between week 2 – week 3 and week 2 – week 4, respectively (Figure 3B). In the HALMETF group a

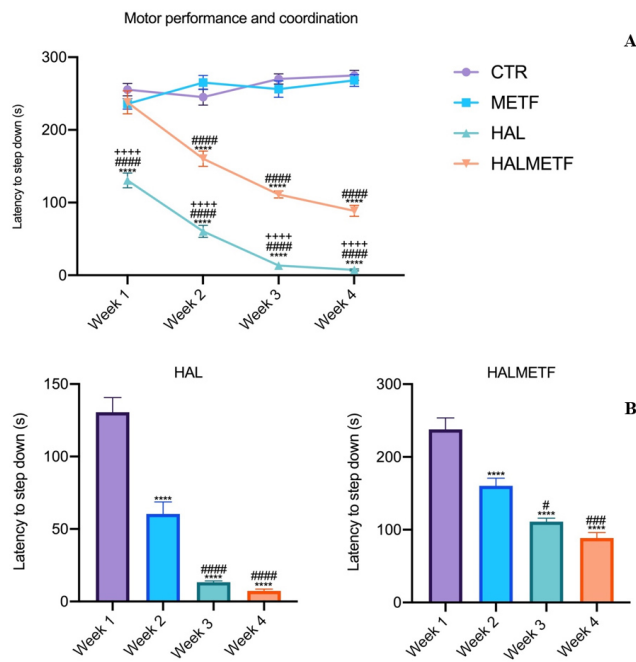


Fig. 3. (A) Motor performance and coordination using rotarod test. Values are expressed as mean ± SEM. Statistically significant differences compared to the CTR group (Control group) are noted with **** $p < 0.0001$. Statistically significant differences compared to the METF group (Metformin group) is noted with #### $p < 0.0001$. Statistically significant differences compared to the HALMETF (Haloperidol + Metformin group) group is noted with ++++ $p < 0.0001$. **(B)** Differences of latency to fall in the rotarod test for group HAL (Haloperidol group) and HALMETF. Results are expressed as mean ± SEM. **** $p < 0.0001$, different from the week 1; # $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$, different from week 2.

slight decrease in motor performance and coordination was observed on week 2 of chronic treatment administration. Moreover, the difference between week 3 and 4 the difference was not significant, suggesting a protective effect of treatment on motor capacity.

Open field

In order to establish the influence of medication (HAL, METF, HALMETF) on motor activity and immobilization time, the OF test was performed. A decrease in locomotor activity in the HAL and HALMETF groups was noted (Figure 4A). The freezing time in OF also demonstrated that the HAL group was the most affected (Figure 4B). However, the HALMETF group showed higher values of distance traveled and decreased time of immobilization.

Novel object recognition

Following the statistical analysis, it was not possible to determine exactly whether the combination with metformin improves the ability to discriminate between objects, so other parameters were also taken into account, such as distance traveled. Following the one-way ANOVA for the determination of DI (Figure 5A), no significant difference was found between the groups included in the study ($F_{3, 26} = 0.1849, p = 0.9057$). A negative DI value was found in the HAL group. In order to calculate the DI, only those

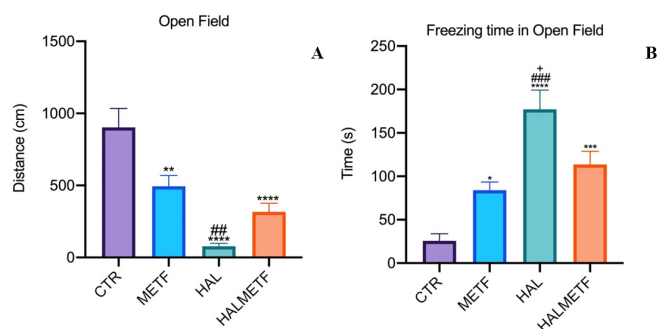


Fig. 4 Locomotor activity and freezing time in the Open Field test. Values are expressed as mean \pm SEM. Statistically significant differences compared to the CTR group (Control group) are noted with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Statistically significant differences compared to the METF (Metformin group) group are noted with ## $p < 0.01$, ### $p < 0.001$. Statistically significant differences compared to the HALMETF group (Haloperidol + Metformin group) are noted with + $p < 0.05$; HAL – Haloperidol group.

animals that showed a cumulative time spent with the two objects of at least one second were considered for analysis (CTR $n = 10$, METF $n = 7$, HAL $n = 5$, HALMETF $n = 8$). Moreover, the freezing time was also evaluated, which reflected the existence of a state of anxiety, manifested in terms of neophobia, as a result of placing the two objects in the test environment. Significant differences were reported between the HAL group compared to the other groups, ($F_{3,36} = 9.435$, $p < 0.0001$), as can be seen in Figure 5B.

To evaluate the motor activity in the Retention trial, the one-way ANOVA test revealed the existence of significant differences between the HAL group and CTR and METF

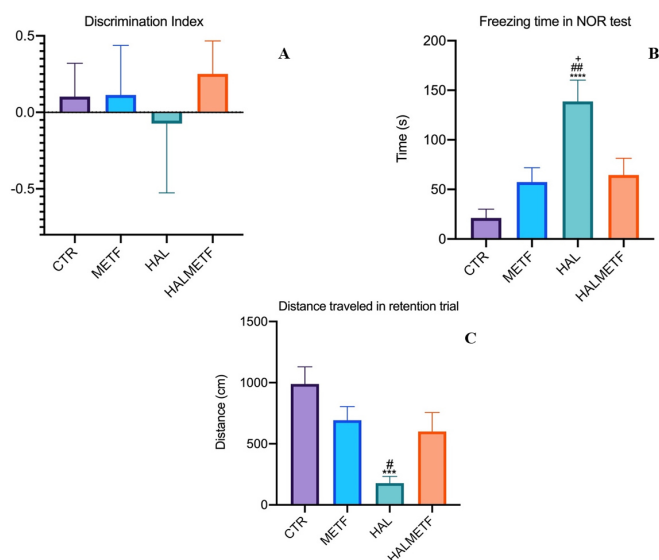


Fig. 5. (A) The effect of chronic treatment with haloperidol and metformin on the discrimination index (DI) of the familiar object and the new object in the retention process, (B) on the period of immobilization during the retention process and (C) on the travel distance in the exploration stage. Data are expressed as mean \pm SEM, *** $p < 0.001$, **** $p < 0.0001$ compared to CTR group, # $p < 0.05$, ## $p < 0.01$ compared to METF group, + $p < 0.05$ compared to HALMETF group. Note that the DI represents the average only of those animals that showed the cumulative curiosity for the two objects, for at least one second (CTR $n = 10$, METF $n = 7$, HAL $n = 5$, HALMETF $n = 8$); HAL – Haloperidol group, HALMETF – Haloperidol + Metformin group.

groups, respectively ($F_{3,35} = 7.584$, $p = 0.0005$), as can be seen in Figure 5C.

Discussion

It is generally accepted that haloperidol, a non-selective dopaminergic receptor antagonist, possess the property of providing an empiric experimental model of Parkinson's disease in rodents due to its anti-dopaminergic mechanism of action and neurotoxicity, favoring bradykinesia and late dyskinesia [31]. However, the most obvious manifestation produced by haloperidol is catalepsy, following D_2 receptor blockade [16, 32].

The results of this study demonstrate an improvement in cataleptic behavior and motor performance induced by haloperidol, following chronic treatment with metformin. Thus, in the catalepsy test, metformin significantly reduced the latency of descent from the bar in all tests performed, regardless of the time. These results are in agreement to those previously presented by *Adedeji et al.* [33], that metformin reduces the period of immobilization and improves the ability of behavioral adaptation [34].

The measurement of motor performance, coordination and balance in the case of the rotarod test, demonstrates a clear deficit in the case of the HAL group, with low latencies of falling off the drum rotating at a constant speed. Another detail worth mentioning is that these small latencies observed after the first week of treatment, are worsening throughout continuation. Interestingly, in the case of metformin, this latency has a less steep slope over time. Indeed, weight and weight gain may have a negative impact on rotarod performance, but rodents were pre-tested and weighed during the accommodation period and body mass remained constant throughout treatment. Therefore, it is unlikely that their performance was affected by weight. Motor dysfunction is obviously associated with the loss of dopaminergic neurons in the nigrostriatal level and dopamine depletion, because these neurons show projections to the striatum and their destruction leads to changes in the neural circuits in the basal ganglia, which are essential in regulating various movements. Thus, the association with metformin shows a significant improvement in motor activity, being consistent with other studies performed on small rodents [35].

In the OF test, there were no significant differences between the groups. However, compared to the HAL group, those who received the drug combination traveled longer distances.

Using the NOR paradigm we tried to assess the cognitive deficit induced by chronic haloperidol treatment and the ability of metformin treatment of preventing it. In addition, a reduction in immobilization time was noted in the presence of the two objects placed in the test environment.

Moreover, based on the influence exerted on the energy metabolism, evidence suggests that the therapeutic potential of metformin comes from the property of regulating the mitochondrial function, which could also affect be

influenced at the central nervous system level (metformin can cross the blood-brain barrier) [13]. Thus, the clinical use of this compound remains to be investigated, and whether it could counteract the neurotoxic effects of haloperidol, such as mitochondrial impairments or disorders of energy metabolism in the brain.

This study confirmed that metformin can improve some of the behavioral alterations induced by haloperidol. Significant attenuation of cataleptic status, improved motor coordination, and attenuation of anxiety-like behavior have been observed. The METF group indicates a shorter immobilization period, in both OF and NOR tests, which could suggest that metformin decreases the anxiety-like behavior, but this hypothesis needs further consideration in future studies [36]. The evaluation of other cognitive functions, such as reversal learning and associative memory, brings additional information in the case of the development of motor and cognitive deficits. The clinical utility of metformin and its influence on energy metabolism and central nervous system function should be investigated in future studies to establish effects in neurological disorders. From the authors perspective, the increase in dosage regimen or in treatment duration would not necessarily lead to an improvement of the analyzed parameters. It is likely that with the increase, the blockage of the complex I of ETC by metformin would occur in a higher measure and the risk of developing lactic acidosis would also increase. Several studies emphasize the reduction of life expectancy on experimental animals [14, 37].

The present study comes with several limitations. First, the animals were individually placed and kept during the experiment, which presumes social isolation induced-stress. Second limitation is related to the fact that the tests were performed during day-time, although it is known that rats are nocturnal animals and their activity is intensified during night-time.

Conclusion

Metformin treatment may have a beneficial effect in haloperidol-induced motor dysfunction in rats, demonstrated by decreased cataleptic behavior and improved rotarod performance. Furthermore, an anxiolytic effect of metformin could also be indicated by a decreased freezing time and increased exploratory behavior, but further studies will be needed for confirmation.

Author's contribution

GJ – Data collection, conceptualization, investigation, writing the original draft, funding acquisition

ZG – Data collection, writing, editing, supervision

CEV – Writing and Editing

BEÖ - Writing and Editing

AMT - Writing and Editing

AG – Writing and Editing

MTD – Writing and Editing, conceptualization, supervision

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Conflicts of Interest

The authors declare no financial or other conflict of interest.

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