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Repeated Exposure to Ozone Produces Changes in Metabolic Disturbances Present in the TDP-43A315T Transgenic Model of Amyotrophic Lateral Sclerosis

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Abstract

Metabolic abnormalities play a key role in the pathogenesis of Amyotrophic Lateral Sclerosis (ALS). Different drugs have been used in the clinical practice; however, no current therapeutic disease-modifying intervention exists. The biological effect of ozone (O_3) has gained much attention in neurodegenerative disorders, due to its strong capacity to induce controlled oxidative stress and inflammation. However, its effect on metabolism are very less studied, even though O_3 is known for cause endocrine and metabolic changes, increasing food intake and body fat mass. Considering that body weight gain and mild obesity appears to improve survival in ALS patients, the aim of this study was to address the role of O_3 on the metabolic disturbances present in ALS. To test this hypothesis, TDP-43^{A315T} mice and age-matched WT littermates, were exposed to O_3 (0.25 ppm) or filtered air (FA) for 15 days (4 hours/day). The effect of O_3 exposure on ALS disease progression was addressed by monitoring body weight loss and motor performance until the disease end-stage in ALS mice. Furthermore, we investigated the action of O_3 on plasma glucose content and biomarkers of metabolism by immunoassay. O_3 exposure significantly improves motor performance and mitigates disease-associated weight loss in TDP-43^{A315T} mice. As well, circulating levels of metabolic proteins and glucose in plasma were highest at disease end-stage after O_3 exposure in TDP-43^{A315T} mice. These findings provide the first insights into the mechanistic link between O_3 exposure and the improvement of the metabolic disturbances present in ALS, based on experimental data.

Keywords: Metabolic disturbances; Pathogenesis; Biomarkers; Neurodegenerative disorders

Introduction

Amyotrophic Lateral Sclerosis (ALS) is a motor neuron disease characterized by the selective and progressive loss of upper and lower motor neurons of the cerebral cortex, brainstem and spinal cord [1]. The result of this loss is a rapidly progressive paralysis, which ultimately leads to death within three to five years of symptom onset. The majority of patients suffer from sporadic ALS (sALS; more than 90%), in which multiple risk factors from gene-environment interactions contribute to the disease pathogenesis. In contrast, only a small subset of patients suffers from familial ALS (fALS; less than 10%) due to their associated genetic dominant inheritance factor [2]. Although the cellular basis for neurodegeneration in ALS is not yet fully understood, numerous studies have shown that the underlying disease process involves multiple complex genetic and non-genetic factors, including metabolic alterations.

Ozone (O_3), is a highly oxidative gas that is formed by the action of solar radiation from photochemical reactions of other pollutants (i.e. nitrogen oxides and volatile organic compounds) emitted by vehicles and manufacturing [3]. Extensive investigation about O_3-related pathophysiological squeals clearly show that exposure to this pollutant causes and exacerbates respiratory and cardiovascular diseases [4-8]. O_3 exposure could also increase the incidence of chronic metabolic disorders as obesity and diabetes type II [9,10]. More recently, a number of studies strongly suggest that certain neurological pathologies, Alzheimer's disease and Parkinson's disease might be associated with the O_3 exposure or lead to a predisposition to these neurological alterations [11-15]. Some environmental and occupational risk factors, including air pollution, could be associated with the occurrence of ALS. However, epidemiological evidence supporting this association is still limited. Further larger studies are needed to assess this relation in order to demonstrate associations between environmental factors, including exposure to O_3, and negative health effects in ALS disease. Indeed, clear evidence on the possible link between air pollution and ALS, based on experimental data, is lacking. Although O_3 has deleterious effects, many therapeutic effects have also been suggested. Paradoxically, being O_3 a natural bioactive molecule which has been traditionally used as a powerful oxidant in medicine, O_3 can trigger biochemical mechanisms involved in the reactivation of the antioxidant system [16]. Recently, the therapeutic potential of O_3 has gained much attention through its strong capacity to induce controlled oxidative stress [17], through mechanism of action involving its interaction with the nuclear factor erythroid-derived 2–like 2 (Nrf2) a key regulator of inducible antioxidant responses [18,19]. A growing new body of clinical data indicates the effectiveness of O_3 as a potential novel strategy to delay

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neurodegeneration, and also with potential effects on immune system and gut microbiota [20,21]. Clinical trials evidenced the effectiveness of O_3 therapy in the treatment of degenerative disorders as Multiple Sclerosis (MS) [18,22,23]. However, no studies have been conducted, so far, to investigate the hypothesis exposure to O_3 might mitigate metabolic disturbances present in ALS, even though O_3 is known for cause endocrine and metabolic changes, and a growing body of evidence shows disturbances in energy metabolism in ALS disease [24-27].

To test this hypothesis, TDP-43^{A315T} mice, a mice model of TDP-43 proteinopathy, and age-matched WT littermates, were exposed to O_3 (0.25 ppm) or Filtered Air (FA) for 15 days (4 hours/day), providing the first insights into the mechanistic link between O_3 exposure and the improvement of the metabolic disturbances present in ALS [28].

Materials and Methods

Animals

Transgenic (Tg) TDP43^{A315T} mice (Strain No. 010700, Bar Harbor, ME, USA) and the genetic background–matched wild type (WT) littermate control mice were used in this study [28]. This mouse model of ALS expresses a mutant human TAR DNA binding protein TDP-43 cDNA harboring an N-terminal flag tag and an TDP43^{A315T} amino acid substitution associated with ALS mainly in the CNS [28]. To avoid ambiguity associated with reported sex-related differences in mean survival time of TDP-43^{A315T} mice, only male mice were used [28, 29]. Animals expressing the hTDP-43 transgene were confirmed *via* PCR according to the distributor's protocol. The ALS-like disease was divided into two stages: onset (defined as the last day of individual peak body weight before a gradual loss occurs) and the end-stage of disease (defined as when weight is 20% below the initial weight on three consecutive days), at which the mice were euthanized. The end-stage is typically reached 2–4 weeks after symptom onset. Animals were group-housed under standard housing conditions with a 12 h light–dark cycle, and food and water ad libitum. To monitor disease onset and progression, all mice were weighed and assessed three times per week until the disease onset-stage, after which they were checked daily in the morning until the disease end-stage. All experimental procedures were approved by the Animal Ethics Committee (AEC) of the National Hospital for Paraplegics (HNP) (Approval No 26/OH 2018) in accordance with the Spanish Guidelines for the Care and Use of Animals for Scientific Purposes. All analyses were conducted by personnel blinded to the animal genotype.

O_3 exposure

42 day old TDP-43^{A315T} mice and WT littermate controls were divided in two groups, and exposed to O_3 (9/group, n=3 TDP-43^{A315T} mice vs. n=6 WT mice) or FA (9/group, n=3 TDP-43^{A315T} mice vs. n=6 WT mice). In all cases, animals were exposed 15 consecutive days (4 hours/day) following the protocol described by Bello-Medina et al. (Figure 1) [11]. O_3 was generated from pure O_2 with a BTM 802N generator and distributed in a Plexiglas chamber (50 × 35 × 35 cm) together with zero air at a total flow of 15 L min-1. The O_3 concentration of the ambient air in the chamber was kept constant at 0.25 ppm and was continuously monitored by an Environment O_3 42 M analyzer (Envea, France). FA was obtained by filtering regular air with activated charcoal to reduce O_3 concentration to a minimum of (<0.02 ppm). During the exposures, animals had food and water ad libitum, and their general health was regularly checked.

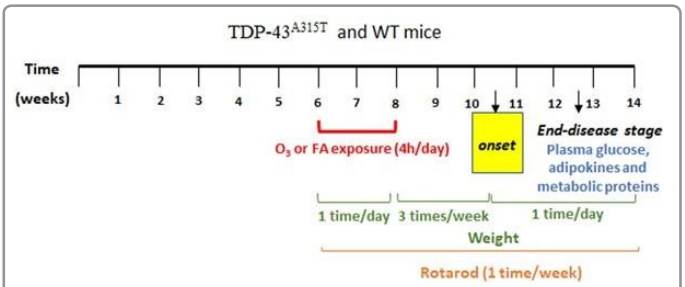

Figure 1: The schedule of experiment design: Male mice about 42 days of age (~ 6 weeks of age) underwent to 15 consecutive days of exposure to O_3 or filtered air (FA). During the exposures (4 hours/day) mice were weighed and assessed daily. After 8 weeks, all mice were weighed 3 times per week until the disease onset-stage (~ 12-13 weeks of age). The rotarod motor test was performed on all mice once a week, starting from the 6 weeks of age until the day of euthanasia (95-100 ± 2 days), when the animals were terminally anesthetized and blood was collected for the measurement of plasma glucose content and metabolic markers using Luminex® 200TM technology.

Monitoring and behavioral assessments

To monitor disease progression and onset determination, body weight lost was measured and motor performance was evaluated using rota rod test. All mice were weighed and assessed three times per week until the disease onset-stage. After that, mice were then checked daily in the morning until the disease end-stage.

The rota rod motor test was performed on all mice once a week, starting from the 6 weeks of age until the day of euthanasia [30] (Figure 1). Unlike SOD1 mouse models of ALS, the TDP-43^{A315T} mice maintained locomotor functionality through to end-stage. Animals were previously trained for three consecutive days and three times a day to promote the learning of the task. The accelerated protocol was applied for this motor monitoring as described previously by Mandillo et al. [31]. In brief, mice were placed on a rota rod apparatus (Model 7650, Ugo Basile) at a speed of 4 rpm with acceleration up to 40 rpm over 300 s. Three tests were performed for each mouse with a minimal interval of 20 mins, and the average of the longest two performances was taken as the final result for analysis.

Measurement of plasma glucose content

At disease end-stage (~95-100 ± 2 days), animals were terminally anesthetized with sodium pentobarbitone (140 mg kg-1) and transcardially perfused with room temperature 0.01 M phosphate buffered saline (PBS; pH 7.4), in the middle of the light cycle (between 11 AM and 1 PM). The whole blood was collected by cardiac puncture [32]. Immediately, glucose levels were measured using a glucometer (FreeStyle Optium™ Neo H glucometer, Abbott Diabetes Care Inc, USA), previously calibrated for plasma glucose levels. The samples remaining after the glucometer measurements were immediately centrifuged at 3380 g for 10 min at room temperature to separate plasma samples, which were immediately frozen on dry ice and stored at -80°C for later analysis [33].

Measurement of metabolic markers plasma

Total ghrelin, the adipokines resistin and leptin, and metabolic biomarkers of insulin resistance (GIP, GLP-1, glucagon, PAI-1 and insulin) from plasma samples were analyzed in duplicate by using the Bio-PlexPro mouse diabetes group from Bio-Rad (Ref. 171F7001M) and Luminex® 200TM technology as previously described [34]. Samples were processed following the manufacturer's instructions. According to Bio-Rad's information the intra and inter assay CV variability is <20%.

The final concentration value of each metabolic marker was the result of the mean from the duplicate measures.

Statistical analysis

All data are presented as Mean ± Standard Error of the Mean (SEM). Differences between means were assessed by two-way ANOVA followed by Tukey post hoc analysis. For multiplex assays, the median for each sample group was determined for all the analytes, and Kruskal–Wallis test was performed followed by Dunett's post hoc test to compare all groups with WT controls in response to FA, while Bonferroni post hoc test were used for multiple comparisons between all groups. Spearman correlation was performed for the adipokines and metabolic proteins, respectively. For all statistical tests, a p value of <0.05 (CI 95%) was assumed to be significant. Statistical analysis was performed using Graph Pad Prism software (version 8.3.1).

Results

Evaluation of O_3 exposure on disease progression

ALS causes loss of body weight and reduced fat mass [35,36]. Thus, as TDP-43^{A315T} mice exhibit weight loss during disease progression [29,37-39], we assessed the capacity of O_3 to modify weight changes over time. During the 15 days of exposures, no differences in weight gain between groups (O_3 group vs. FA group) were displayed (Figure 2). A two-way ANOVA with repeated measures revealed a significant genotype interaction (Figure 3A; $F(15, 81)=87.72$, $p<0.0001$), indicating a sustained decline in body weight in TDP-43^{A315T} mice compared to WT controls in responses to FA or O_3 over time. Although there was a trend, no significant differences were observed between FA-exposed or O_3-exposed TDP-43^{A315T} mice, however, the calculation of the disease onset using this parameter indicated that TDP-43^{A315T} O_3-exposed develop symptoms later than FA-exposed TDP-43^{A315T} mice. Using body weight measurements an average onset of 75 ± 5 days of age was determined for in TDP-43^{A315T} mice in responses to FA, whereas in responses to O_3, TDP-43^{A315T} mice presented a phenotype at 83 ± 1 days of age (Figure 3B). To further assess changes in weight due to the effect of O_3 exposure on disease progression based on the determined disease onset, we calculated disease duration (Figure 3C). Comparatively the disease duration was longer in TDP-43^{A315T} mice in responses to O_3(Figure 3C), although O_3-exposed TDP-43^{A315T} mice did not live significantly longer compared with FA-treated TDP-43^{A315T} mice.

We also tested motor behavior to determine if O_3 exposure could alter disease phenotype. Our results indicate a progressive decline in motor coordination in TDP-43^{A315T} mice, confirming the progressive motor deficits of the TDP-43^{A315T} mouse model reported previously [40]. TDP-43^{A315T} mice consistently scored lower than WT littermate controls at all time-points examined. A two-way ANOVA with repeated measures revealed a significant interaction of the group by week (Figure 3D) ($F(6,97)=3.051$ $p<0.008$), indicating differential change over time in rotarod performance. However, O_3-exposed TDP-43^{A315T} mice showed a significant improvement in motor performance at later time points, indicating the potential effect of O_3 on motor function. Indeed, the rotarod test displayed that FA-exposed TDP-43^{A315T} mice suffer a more significant drop in performance and progressive impairment in motor capacity over time.

Evaluation of O_3 exposure in adipokines and metabolic proteins

We next studied how adipokines and proteins involved in metabolism were affected in the plasma of TDP-43^{A315T} mice at the end-stage of disease (Figure 4). We found altered circulating grelyn, resistin and leptin concentrations in the plasma of TDP-43^{A315T} mice compared to age-matched controls (Figures 5A-5C). Dunett's post hoc test demonstrated a significant reduction in the plasma levels of resistin in FA-exposed TDP-43^{A315T} mice compared to FA-WT controls (Figure 5B). Bonferroni's post hoc test demonstrated a significant reduction in the circulating levels of resistin in FA-exposed TDP-43^{A315T} mice compared to O_3-exposed WT controls (Figure 5B). Interestingly, although a significant reduction in the levels of leptin in TDP-43^{A315T} mice exposed to either O_3 or FA compared to O_3-exposed WT mice, no significant differences were determined in the circulating levels of leptin in O_3-exposed WT mice compared to FA-exposed WT controls (Figure 5C).

To further analyze metabolism, circulating levels of PAI-1, GIP, GLP-1, insulin and glucagon peptides were measured in TDP-43^{A315T} and age-matched WT littermates at the end-stage of disease (Figures 5D-5H) [41]. Circulating plasma levels of PAI-1, GIP, GLP-1, and insulin are higher in O_3-exposed TDP-43^{A315T} mice relative to WT controls (Figures 5D-5G), while no statistical difference were determined compared to either O_3 or FA WT mice. Finally, no linear correlation was found using Spearman's test among the among the plasmatic levels of total ghrelin, and the adipokines resistin and leptin, in WT controls (Figure 6A) or TDP-43^{A315T} mice (Figure 6B) exposedto FA or O_3, respectively. Nevertheless, it is worth noting that a positive linear correlation was found using Spearman's test among the plasmatic levels of GIP and insulin (95% CI [0.027, 0.950], p=0.039) as well as insulin and glucagon (95% CI [0.043, 0.951], p=0.036) concentrations in WT controls (Figure 7A), although no linear correlation was found compared to TDP-43 mice (Figure 7B).

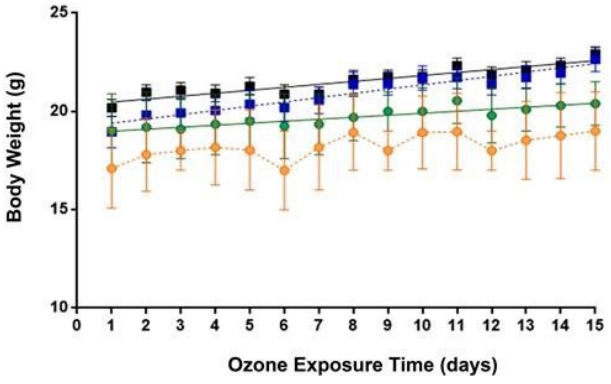

Figure 2: O_3 exposure beginning at the asymptomatic state of disease does not alter body mass in TDP-43^{A315T} mice. Body weight was monitored daily in WT controls and TDP-43^{A315T} mice exposed to FA or O_3 for 15 days, beginning at the asymptomatic state, at 42 days of age (~ 6 weeks of age). No differences in weight gain between groups were determined. Straight line is a regression line, and values are expressed as a mean ± SEM. Comparison between groups was performed by two-way ANOVA, where * $p<0.05$ vs. FA-exposed WT control mice; #$p<0.05$ vs. O_3-exposed WT control mice; $p<0.05 vs. O_3-exposed TDP-43^{A315T} mice. (—■—) Control FA; (—■—) Control O_3; (—●—) TDP- 43 FA; (—●—)TDP -43 O_3

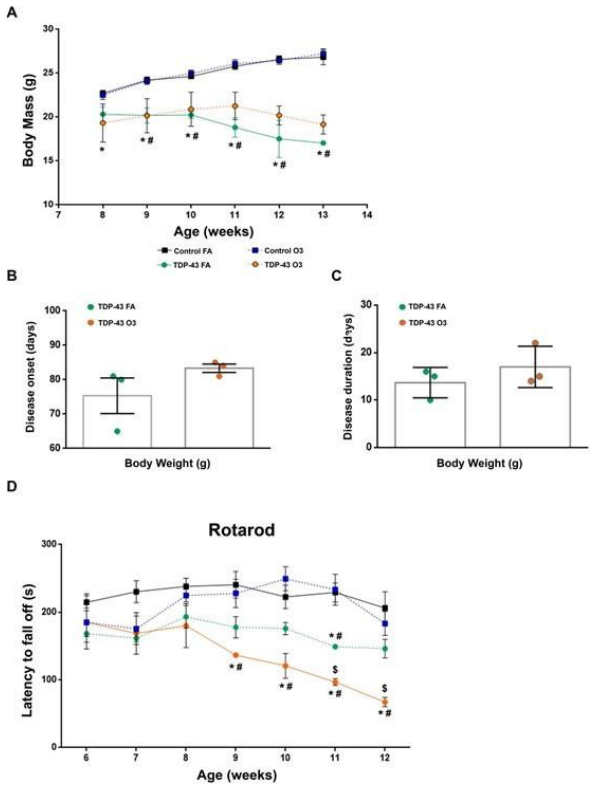

Figure 3: O_3 exposure beginning at the asymptomatic state of disease does not alter body mass change but significantly improves motor performance in TDP-43^{A315T} mice. ( ) Control FA; ( ) Control O_3; ( ) TDP- 43 FA; ( ) TDP-43 O_3

(3A) Body weight was monitored over time in WT controls and TDP-43^{A315T} mice exposed to FA or O_3 Starting weight on week 8. No significant differences were observed between FA-exposed or O_3-exposed TDP-43^{A315T} mice. (3B) Average disease onset and disease duration. (3C) was determined in WT controls and TDP-43^{A315T} mice exposed to FA or O_3 using body weight as a physiological parameter. Average disease duration of the animal was calculated as the time between the onset of disease (defined as the last day of individual peak bodyweight before gradual loss occurs) and the day of death. Comparatively the disease duration was higher in TDP-43^{A315T} mice in responses to O_3. (3D) Behavioral assessment of motor function was performed in WT controls and TDP-43^{A315T} mice exposed to FA or O_3 over time. Significant differences between FA- and O3-exposed mice were seen. Values are expressed as a mean ± SEM. Comparison between groups was performed by two-way ANOVA, where *p<0.05 vs. FA-exposed WT control mice; #p<0.05 vs. O3-exposed WT control mice; $p<0.05 vs. O_3-exposed TDP-43^{A315T} mice. Abbreviations: Control; non-transgenic littermates wild-type (WT), TDP-43; TDP43^{A315T} mice; FA, filtered air, O_3 ; ozone. Corresponding graphs as per A, i.e. control-FA (n=6, black square and solid line), control-O (n=6, blue square and dashed line), TDP-43^{A315T}-FA (n=3, green circ3les and solid line), TDP-43$^{A315T-O}$ (n=3, orange circles and 3 dashed line).

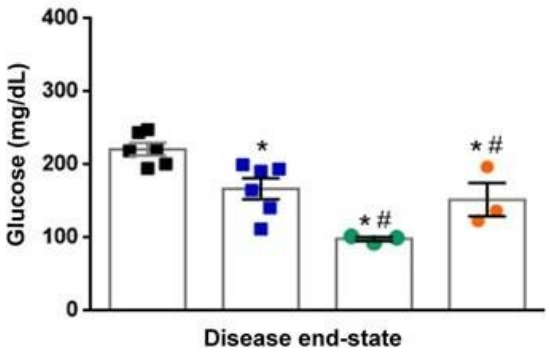

Figure 4: O_3 exposure beginning at the asymptomatic state increased plasma glucose levels in TDP-43A315T mice. Plasma glucose concentration was measured in WT controls and TDP-43A315T mice exposed to FA or O_3 at disease end-stage (~ 95-100 ± 2 days). Quantification revealed the highest levels in response to O_3 exposure in TDP-43A315T mice. In addition, significant differences between FA- and O_3-exposed Control-mice were seen. Values are expressed as a mean ± SEM. Comparison between groups was performed by two-way ANOVA, where *p<0.05 vs. FA-exposed WT control mice; # p<0.05 vs. O_3-exposed WT control mice. ( ) Control FA; ( ) Control O_3; ( ) TDP- 43 FA; ( ) TDP-43 O_3

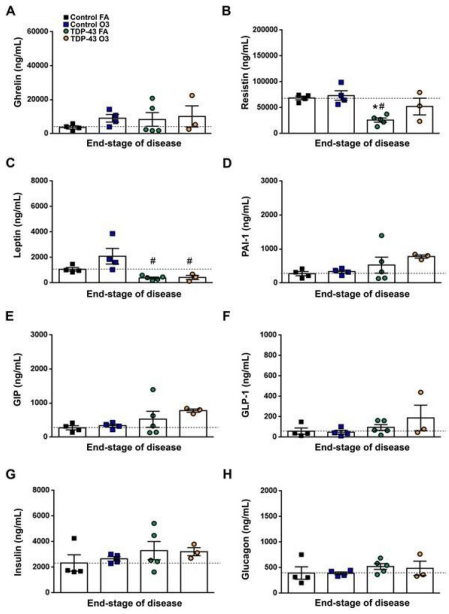

Figure 5: Adipocytokines and metabolic biomarkers levels in WT controls and TDP-43^{A315T} mice exposed to FA or O_3. Total ghrelin, plasma adipokines (resistin and leptin) and metabolic biomarkers of insulin resistance (GIP, GLP-1, Glucagon, PAI-1 and insulin) was measured in WT controls and TDP-43^{A315T} mice exposed to FA or O_3 at disease end-stage (~ 95-100 ± 2 days) using Luminex® 200TM technology. Values are expressed as mean ± SEM for the different groups: Control FA (black square, n=4), Control O_3 (blue square, n=4), TDP-43^{A315T} FA (green circles, n=5) and TDP-43^{A315T} O_3 (orange circles, n=3). Kruskal–Wallis test was performed followed by Dunett's post hoc test to compare all groups with WT controls in response to FA, while Bonferroni post hoc test were used for multiple comparisons between all groups. (A) Ghrelin plasmatic concentration.(B) Resistin levels. *p<0.01 vs. Control FA; # p<0.005 vs. Control O_3. (C) Leptin levels. # p<0.05 vs. Control O_3. (D) PAI-1 levels. (E) GIP levels. (F-H) GLP-1, insulin, and glucagon plasmatic levels, respectively.

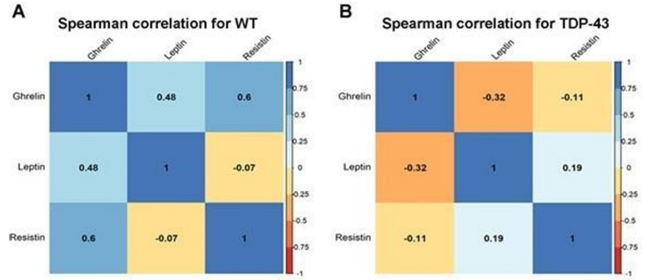

Figure 6: Spearman correlations for the adipocytokines ghrelin, resistin and leptin in WT controls and TDP-43^{A315T} mice exposed to FA or O_3 In this figure, the red and blue squares refer to negative and positive correlations, respectively. The color intensity of the squares is proportional to the correlation coefficient. In the legend at the right size, the intensity of the color shows the rate of correlations and the corresponding relationships.

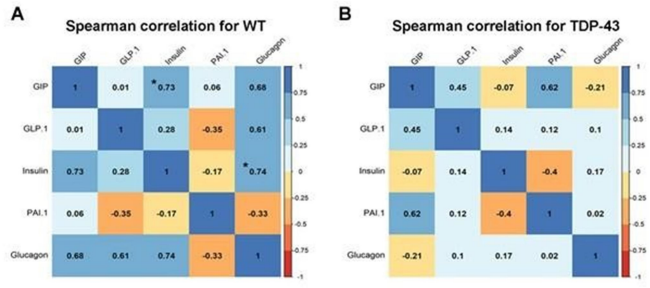

Figure 7: Spearman correlations for metabolic proteins in WT controls and TDP-43A315T mice exposed to FA or O_3. In this figure, the red and blue squares refer to negative and positive correlations, respectively; *p<0.05. The colour intensity of the squares is proportional to the correlation coefficient. In the legend at the right size, the intensity of the colour shows the rate of correlations and the corresponding relationships.

Discussion

A growing body of evidence indicates disturbances in energy metabolism in ALS, suggesting that targeting metabolism could represent a rational strategy to treat this disease [24-27]. Metabolic abnormalities have been reported in both ALS patients and mouse models of ALS [42-45]. A new body of clinical data indicates the effectiveness of O_3 therapy in the treatment of degenerative disorders; however, no studies have been conducted, so far, to investigate the direct influence of O_3 in altering energy metabolism and disease progression in ALS [18, 22, 23]. Here, we present evidence of the effect of O_3 on motor behavior and disease-associated weight loss in the TDP43^{A315T} transgenic ALS mouse model, providing novel insights about the mechanistic link between O_3 exposure and the improvement of the metabolic disturbances present in ALS disease.

Here we report that O_3 exposure has an impact on the regulation of the expression of certain metabolic proteins that are linked to metabolic disease (i.e. obesity and diabetes type II). This is concordant with previous mouse studies that saw that acute O_3 exposure causes endocrine and metabolic changes, increasing food intake and body fat mass [46]. We have identified specific alterations in circulating levels of GIP, PAI-1, and insulin in response to O_3 exposure in TDP-43^{A315T} mice. This observation is of interest because it has been shown that PAI-1 and insulin are metabolic proteins associated with an increase in adiposity and Body Mass Index (BMI), and GIP stimulates insulin secretion in response to food intake [47-49]. These data might indicate that O_3, cause endocrine and metabolic changes in TDP-43^{A315T} mice which would help reduce disturbances in energy metabolism associated with the progression of ALS. Indeed, O_3 exposure mitigates the sustained decline in body weight in TDP-43^{A315T} mice over time. This observation is of interest because patients with ALS are unable to maintain their body weight and rapid weight loss in ALS disease is associated with worse disease outcomes [50-52]. In this context, it would be plausible that this effect of O_3 on body weight gain in TDP-43^{A315T} mice could partly be due to modifications in food intake and ultimately the loss of body weight in TDP-43^{A315T} mice [29,37-39]. Indeed, a positive correlation was found among the plasmatic levels of GIP and glucagon relative to insulin concentrations in WT controls, which strongly associated exposure to O_3 with the development of obesity [53]. However, future experiments should try to corroborate this hypothesis.

Furthermore, although a progressive motor impairment has been described in TDP-43^{A315T} mice [40], our results confirm that O_3 significantly improved motor function in TDP-43^{A315T} mice over time, and resulted in higher plasma glucose levels at end-stage of ALS disease. The observation in glucose content in plasma is of particular interest as ALS patients have glucose metabolism defects which could partly be due because of higher levels of circulating glucagon [54]. Indeed, we found lower levels of circulating glucagon in O_3-exposed TDP-43^{A315T} mice compared to FA-treated TDP-43^{A315T} mice, and a positive correlation was determined among the plasmatic levels of GIP and glucagon compared to insulin concentrations in WT mice. Thus, since it has been suggested that an increase in disease severity in ALS patients is correlated with increased circulating glucagon levels our data are congruent with the observations that insulin resistance is related to disease severity and outcome in ALS [55].

Conclusion

In summary, our study provides the first experimental evidence suggesting for a potential beneficial effect of O_3 in motor function and some metabolic disturbances present in TDP-43^{A315T} mice. However, the precise pathways that could link O_3 effects to the TDP-43 proteinopathy model of ALS remain unclear. Further mechanistic studies analyzing how O_3 contributes to disease progression may require at the inclusion of additional defined time-points, as well as larger sample sizes. Determining the mechanistic actions of O_3 may provide a new avenue for therapeutic development for this fatal condition.

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Conflicts of Interest

The authors declare no conflict of interest.

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