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ORIGINAL ARTICLE

Nitrous oxide as an adjunctive therapy in major depressive disorder: a randomized controlled double-blind pilot trial

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Objective: Major depressive disorder (MDD) is related to glutamatergic dysfunction. Antagonists of glutamatergic N-methyl-D-aspartate receptor (NMDAR), such as ketamine, have antidepressant properties. Nitrous oxide (N_2O) is also a NMDAR antagonist. Thus, this study aimed to evaluate the effects of augmenting antidepressant treatment with N_2O.
Methods: This double blind, placebo-controlled randomized parallel pilot trial was conducted from June 2016 to June 2018 at the Hospital das Clínicas, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo. Twenty-three subjects with MDD (aged 18 to 65, on antidepressants, with a score $>$ 17 on the 17-item-Hamilton Depression Rating Scale [HAM-D_{17}]) received 50% N_2O (n=12; 37.17$\pm$13.59 years) or placebo (100% oxygen) (n=11; 37.18$\pm$12.77 years) for 60 minutes twice a week for 4 weeks. The primary outcome was changes in HAM-D_{17} from baseline to week 4.
Results: Depressive symptoms improved significantly in the N_2O group (N_2O: from 22.58$\pm$3.83 to 5.92$\pm$4.08; placebo: from 22.44$\pm$3.54 to 12.89$\pm$5.39, $p < 0.005$). A total of 91.7% and 75% of the N_2O group subjects achieved response ($\geqslant$ 50% reduction in HAM-D_{17} score) and remission (HAM-D_{17} $<$ 7), respectively. The predominant adverse effects of N_2O treatment were nausea, vomiting, and headache.
Conclusion: N_2O treatment led to a statistically significant reduction in HAM-D_{17} scores compared to placebo.
Clinical trial registration: Brazilian Register of Clinical Trials, RBR-5rz5ch

Keywords: nitrous oxide; major depressive disorder; glutamatergic system; NMDA receptor.

Introduction

Major depressive disorder (MDD) is the fifth leading cause of disability worldwide, accounting for 4.2% of disability-adjusted life years lost. Taking into account only non-communicable diseases, depressive disorders are the second leading cause of disability worldwide.[1] The average 12-month prevalence of MDD is 4.7%, ranging from 1.1% in Nigeria to 10.1% in Brazil, whereas the mean lifetime prevalence is 11.2%.[2] More than half of patients have a recurrent or chronic course. They also have reduced quality of life, increased mortality and morbidity associated with other chronic medical conditions, as well as increased suicide rates.[3] In addition, depressive disorders cause a high economic burden.[4,5]

The goals in MDD treatment include complete remission of symptoms and a return to premorbid functioning and quality of life.[3] Since the discovery of the antidepressant agents in the 1950s, pharmacological treatment has been the main approach to MDD.[6] The monoaminergic hypothesis was based on the mechanism of action of these first generation antidepressants, postulating that depressive symptoms were a consequence of dysfunctions in monoaminergic neurotransmitter systems. Although roughly 80% of patients taking antidepressants achieve a response after four treatments,[7,8] only 25-40% reach complete remission.[9] Moreover, there is a delay of days to weeks for the therapeutic response to modern antidepressants, which prolongs the patient's suffering and burden.[10] These limitations have led researchers to question the central role attributed to monoamines in depression.[11]

Glutamate is the main excitatory neurotransmitter in the central nervous system, and the glutamatergic system is

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essential for neuronal plasticity, synaptogenesis, and excitotoxicity.[12] Trullas & Skolnick[13] demonstrated that antagonists of N-methyl-D-aspartate subtype receptors (NMDAR) of glutamate reverse the behavioral inhibition induced by animal models of depression. The authors then hypothesized that pathways regulated by NMDAR would be involved in the pathophysiology of depression. Later, based on pre-clinical studies that followed the pioneering study by Collingridge et al.,[14] Berman et al.[15] demonstrated that an intravenous infusion of 0.5 mg/kg ketamine (an NMDAR antagonist anesthetic) had rapid-onset antidepressant effects.

Preclinical studies have focused on other possible mechanisms underlying the antidepressant effects of ketamine, beyond its action on NMDAR. Some of the mechanisms suggested to be involved in its effects include activation of the mammalian target of the rapamycin pathway, activation of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors,[16] inhibition of glycogen synthase kinase 3, and inhibition of eukaryotic elongation factor 2.[11] All of these actions converge to synaptic density protein synthesis, which is necessary for dendritic and somatic plasticity.[16]

Like ketamine, nitrous oxide (N_2O) is an anesthetic that acts as an NMDAR antagonist, which could indicate a possible antidepressant effect. The main mechanism proposed for the anesthetic action of N_2O is the inhibition of glutamatergic excitatory neurotransmission through noncompetitive NMDAR antagonism.[17] The analgesic action of N_2O occurs in part by stimulating the release of endogenous opioid peptides in the midbrain.[18] In addition, N_2O plays a direct and partial agonist effect on mu, kappa, and delta opioid receptors. Regarding its anxiolytic effects, one putative mechanism is the activation of gamma-aminobutyric acid type A receptors at the benzodiazepine binding site. Another mechanism proposed for the anxiolytic effect of N_2O is its action on serotonin neurotransmitters. A study in rats showed increased serotonergic activity in the hypothalamus and decreased activity in the cerebral cortex with the administration of N_2O, the latter possibly as a result of inhibition of pre-synaptic 5-HT_{1A} serotonin autoreceptors.[19]

N_2O is safe and free of serious adverse reactions when used in the recommended therapeutic concentrations.[20,21] The main caveat concerns the interference in homocysteine metabolism through inactivation of cobalamin (vitamin B12). Vitamin B12 deficiency may result in megaloblastic anemia, leukopenia, myelopathy, memory impairment, and behavioral changes.[22]

In 2015, Nagele et al.[23] published a proof-of-concept trial evaluating the effect of N_2O inhalation in a placebo-controlled, double-blind, crossover trial. Twenty treatment-resistant depressive patients received a single administration of 50% N_2O for 60 minutes. N_2O was superior to placebo in improving depressive symptoms (a reduction of 4.8 points in 2 hours and 5.5 points in 24 hours on the 21-item-Hamilton Depression Rating Scale). N_2O was associated with a response rate of 20%, compared to 5% with placebo. No serious adverse events were reported. In their trial, only treatment-resistant patients were enrolled, and a single N_2O treatment was administered. Assessments were made 2 and 24 hours after N_2O inhalation. The authors indicated the need for further trials to determine optimal dosing strategies and to evaluate a more diverse population of patients.

Taken together, the available data suggest that the glutamatergic system is involved in the neurobiology of depression by modulating NMDAR and other targets in this system. Therefore, glutamatergic modulators appear to have antidepressant potential. Thus, we hypothesized that N_2O could augment the effects of antidepressant treatment and conducted a 1-month randomized controlled pilot trial to investigate the efficacy of N_2O in reducing depressive symptoms among MDD patients currently undergoing treatment who remained symptomatic.

Methods

This double-blind placebo-controlled randomized parallel-group (1:1) pilot trial was conducted at the Laboratório de Psicofarmacologia, Hospital das Clínicas, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo (HCFMRP-USP).

The eligibility criteria from the initial trial protocol required two adjustments to increase the number of eligible subjects. Instead of including only subjects on selective serotonin reuptake inhibitors, we decided to include subjects using any class of antidepressant, alone or in combination, in addition to reducing the period without antidepressant dose adjustment from 6 to 4 weeks. The remaining criteria were maintained.

Eligible participants were men or women aged between 18 and 65 years who had been diagnosed with MDD according to DSM-5 criteria, were on antidepressant treatment without dose adjustments for at least 4 weeks, and who scored $>$ 17 on the 17-item-Hamilton Depression Rating Scale (HAM-D_{17}). Exclusion criteria consisted of 1) diagnosed bipolar, psychotic, or substance use disorder (except caffeine and nicotine), a clinical history of antidepressant-induced hypomania/mania episodes; 2) psychotic symptoms; 3) significant suicidal ideation; 4) chronic lung diseases or other relevant clinical conditions; 5) pregnancy or breastfeeding; 6) contraindications to the use of N_2O (chronic vitamin B12 deficiency, pneumothorax, elevated intracranial pressure, intestinal obstruction); and 7) difficulty breathing through the nose. Participants were recruited from the outpatient services of the university hospital, referred by private clinics, or spontaneously sought out the trial through a public advertisement campaign.

Interventions

Subjects were randomly allocated to receive either N_2O or placebo. N_2O was administered at a concentration of 50% diluted in 50% oxygen for 60 minutes in eight sessions (twice a week over 4 weeks). Intervals between trial sessions were 3 or 4 days, alternately. We chose the 50% concentration due to its safety for outpatient administration, since this concentration produces minimal sedation and has no impact on cardiorespiratory function, according to American Anesthesiology Association guidelines.[24] Furthermore, Nagele et al. used this concentration in their

pilot trial[23] N_2O was inhaled through a disposable nasal mask coupled to a portable device for nitrous oxide/oxygen sedation and analgesia (Mandala-Matrix MDM, Porter Instruments, Hatfield, PA, USA). The air volume was between 5 and 7 L/min, which is the respiration volume for a healthy adult.[25]

The placebo condition consisted of 100% oxygen inhaled for the same period through a nasal mask coupled to the same device. This was chosen based on the limitations of our equipment and for blinding purposes. Evidence suggests that 1 hour of normobaric 100% O_2 is safe, and central nervous system toxicity does not occur during normobaric exposures.[26-28] Physiological parameters (heart rate, oxygen peripheric saturation, and blood pressure) were continuously monitored during the session as a safety measure. The sessions lasted approximately 3 hours, including the inhalation period (N_2O or oxygen) and the clinical assessment.

The sessions were performed individually under the supervision of the staff anesthesiologist. For safety issues, the subjects were instructed not to eat food or drink liquids (except water) for at least 2 hours before each session. As an additional safety measure, women of reproductive age took a urinary pregnancy test during the first session before beginning the study procedures.

Outcomes

Changes in HAM-D_{17} scores from baseline to the end of week 4 were considered the primary outcome. Secondary outcomes included changes in Beck Depression Inventory-II (BDI-II) scores from baseline to the end of week 4, therapeutic response and remission at the end of the trial according to HAM-D_{17} and BDI-II scores, the effects on suicidal ideation, which were assessed with the Columbia-Suicide Severity Rating Scale (C-SSRS), the occurrence of manic symptoms, which was assessed with the Young Mania Rating Scale (YMRS), and any adverse effects reported by the subjects or observed by the clinicians. All measurements were taken by a psychiatrist at the beginning (pre-inhalation) and end (post-inhalation) of each session, totaling 16 evaluations throughout the trial.

Randomization and blinding

For subject allocation, a randomization plan was generated at Randomization.com, with randomly permuted blocks in a 1:1 ratio. The subjects and the psychiatrist in charge of the assessments were kept blind to group allocation. Two collaborators not blinded to group allocation were responsible for setting the equipment parameters to provide 50% N_2O or 100% oxygen according to the randomization plan while the psychiatrist in charge of the assessment was absent from the room. This procedure was done at the beginning and end of the 60-minute inhalation period. Other measures to strengthen blinding included covering the device's digital display with a dark cloth and masking the slightly sweet odor of N_2O by using flavored nasal masks.

Statistical analysis

Statistical analysis was performed in SPSS version 23.0. Clinical and demographic characteristics were compared through non-parametric analysis for categorical data (χ^2 test), and a parametric test was used for normally-distributed nominal data (*t*-test for independent samples).

To assess the effects of adjuvant N_2O or placebo to antidepressant treatment, we performed repeated measures analysis of variance for HAM-D_{17} scores considering the factors time, drug (N_2O vs. placebo), and time-drug interaction. When significant time-drug interactions were detected, independent sample *t*-tests were applied for each evaluation point. HAM-D_{17} scores were also analyzed for each group separately using repeated-measures analysis of variance and paired *t*-tests with pre-inhalation and post-inhalation data from each session.

To assess the effect size of the differences found, we used η partial square (η^2_p) tests and classified values according to Maroco's method.[29] In this classification table, η^2_p values > 0.5 correspond to very high effect size, 0.25-0.5 to high effect size, 0.05-0.25 to medium effect size, and $\leqslant 0.05$ to small effect size. The same analyses were performed for BDI-II and YMRS data.

The severity of suicidal ideation was assessed using the ordinal subscale of the C-SSRS and non-parametric Mann-Whitney *U* tests to detect differences between the two groups at each assessment point.

As an additional analysis, we calculated the number of subjects in each group who fulfilled criteria for remission and therapeutic response at the end of the trial. We compared the rates of both groups using Fisher's exact test. For the HAM-D_{17}, remission was defined as scores < 7 and therapeutic response as reductions $\geqslant 50\%$ against baseline scores. For the BDI-II, remission was defined as scores $\leqslant 13$ and therapeutic response as reductions $\geqslant 50\%$ against baseline scores.

Ethics statement

The subjects were given a complete description of the trial and written informed consent was obtained from those who agreed to participate. The study was conducted in accordance with the terms of the declaration of Helsinki, approved by the HCFMRP-USP board of ethics (CAAE: 46352015.5.0000.5440), and registered in the Brazilian Register of Clinical Trials (http://www.ensaiosclinicos.gov.br; number RBR-5rz5ch).

Results

Subjects were recruited between June 2016 and June 2018, and the trial sessions were held between August 2016 and June 2018. A total of 201 subjects were assessed for eligibility, of whom 23 were included in the final sample. Eleven subjects were randomly allocated to the placebo group and 12 were allocated to the N_2O group. Two subjects (both female) in the placebo group did not complete the eight sessions of the trial. One subject left the trial after the first session, reporting worsening of

symptoms. The other subject was excluded after the fifth session due to poor adherence to the treatment protocol because of difficulties leaving home (Figure 1).

The 23 subjects who began the trial were included in the analysis of demographic and baseline clinical characteristics. For the analysis of outcome measures at the end of the trial, only the 21 subjects who completed the eight sessions were considered. The exclusion of these subjects did not affect group matching.

Demographic and clinical characteristics

There were no significant differences between the two groups regarding age, sex, and severity of symptoms according to the HAM-D_{17} (with scores corresponding to a moderate episode in both groups) or the BDI-II (with scores corresponding to a severe episode in both groups). The demographic and clinical characteristics of the subjects are presented in Table 1.

HAM-D_{17}

Significant reductions in HAM-D_{17} scores occurred over time in both groups, with very high and high effect sizes in the N_2O and placebo groups, respectively (N_2O: $F_{5,\ 165} = 15.33$; $p < 0.001$; $\eta^2_p = 0.58$ and placebo: $F_{15,\ 120} = 5.15$; $p < 0.001$; $\eta^2_p = 0.39$). Score reductions in the N_2O group were greater than those in the placebo group (mean $\pm$ standard deviation [SD]: from 22.58 ± 3.82 to 5.92 ± 4.08 and from 22.44 ± 3.54 to 12.89 ± 5.39,

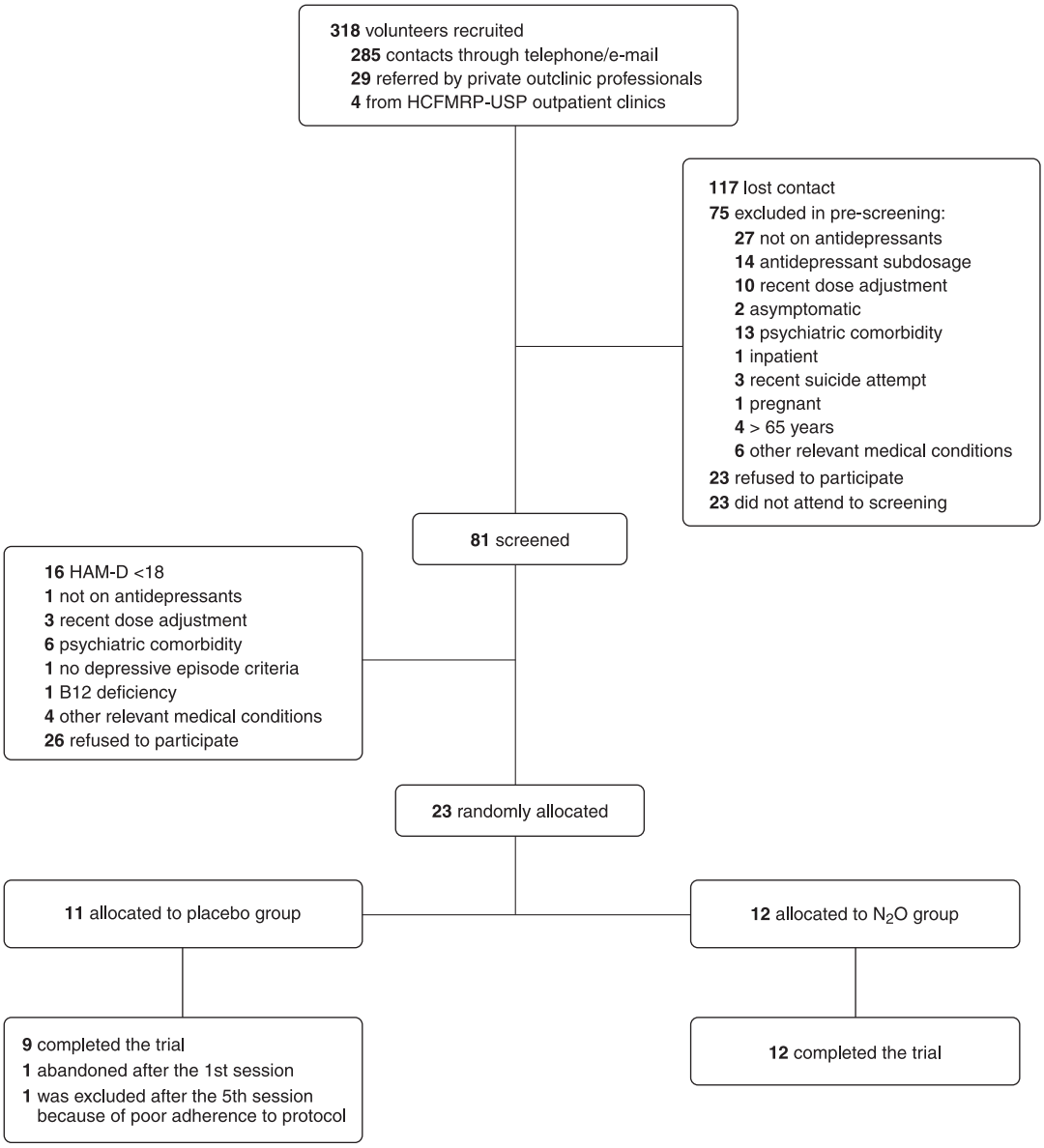

Figure 1 Flow diagram describing the trial's sample recruitment steps and group allocation. HCFMRP-USP = Hospital das Clínicas, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo; HAM-D = Hamilton Depression Rating Scale; N_2O = nitrous oxide.

Table 1 Baseline demographic and clinical characteristics

Variable	Placebo (n = 11)	N_2O (n = 12)	Statistics	p-value
Age (years)	37.18±12.77	37.17±13.59	$t_{21} = 0.003$	1.00
Sex female/male	9 (82)/2 (18)	10 (83)/2 (17)	$\chi^2 = 0.01$	0.92
Baseline HAM-D_{17}	22.58±3.82	22.64±3.77	$t_{21} = 0.03$	0.97
Baseline BDI-II	37.25±9.76	37.54±5.63	$t_{21} = 0.09$	0.93
Number of depressive episodes				
1	2 (18)	1 (8)	-	-
⩾ 2	9 (81)	11 (92)	-	-
Psychiatric comorbidities				
GAD	4 (36)	5 (42)	-	-
OCD	0 (0)	1 (8)		
Psychotropics in use (monotherapy)	3 (27)	3 (25)	-	-
Antidepressants				
SSRI	7 (64)	8 (67)	-	-
SNRI	4 (36)	4 (33)	-	-
Bupropion	2 (18)	2 (17)	-	-
Tricyclic	1 (9)	0 (0)	-	-
Quetiapine	1 (9)	1 (8)	-	-
Lamotrigine	1 (9)	0 (0)	-	-
Topiramate	1 (9)	1 (8)	-	-
Lithium	1 (9)	0 (0)	-	-
Benzodiazepine	6 (54)	4 (33)	-	-

Data are presented as means ± standard deviation or n (%).
BDI-II = Beck Depression Inventory-II; SSRI = selective serotonin reuptake inhibitor; SNRI = noradrenaline and serotonin reuptake inhibitor; N_2O = nitrous oxide; GAD = generalized anxiety disorder; OCD = obsessive-compulsive disorder; HAM-D_{17} = 17-item Hamilton Depression Rating Scale.

respectively). Group comparisons showed a significant time-drug interaction effect with a high effect size ($F_{15, 285}$ = 2.26; p = 0.005; η^2_p = 0.11). Independent sample *t*-tests for each of the 16 assessments throughout the trial showed significant differences with the post-inhalation assessment at the fourth session, although this effect disappeared in sessions 5 and 6 until reappearing in the final two sessions, as shown in Figure 2.

Within each session, paired *t*-tests showed significant reductions in post- vs. pre-inhalation scores in all sessions in both groups, except for the sixth session of the placebo group. However, considering the group means, only the N_2O group reached the remission range, which occurred in the post-inhalation assessment of the seventh and eighth sessions. Likewise, only the N_2O group reached means within the therapeutic response range compared to baseline scores, which occurred in the post-inhalation assessment of the third and fourth sessions, and again in the sixth, seventh, and eighth sessions.

At the end of the eighth session, 75% (9 of 12) of subjects in the N_2O group achieved remission vs. 11.1% (1 of 9) in the placebo group, a difference of 0.64 (p = 0.008; Fisher's exact test). The response rates were 91.7% (11 of 12) in the N_2O group vs. 44.4% (4 of 9) in the placebo group, a difference of 0.47 (p = 0.046; Fisher's exact test).

BDI-II

Significant reductions in BDI-II scores with large effect sizes occurred in both groups (N_2O: $F_{15,165}$ = 8.15, p < 0.001, η^2_p = 0.43; placebo: $F_{15, 120}$ = 6.03, p < 0.001, η^2_p = 0.43). Score reductions were greater in the N_2O group (N_2O: from 37.25±9.76 to 13.75±12.04; placebo: from 36.33±4.92 to 16.33±9.22); however, there were no time-drug interaction effects ($F_{15, 285}$ = 1.12, p = 0.34, η^2_p = 0.06). At the end of the trial, 66.7% of subjects in the N_2O group (8 of 12) reached remission criteria vs. 33.3% (3 of 9) in the placebo group, with a non-significant difference of 0.33 (p = 0.2; Fisher's exact test). The response rates were 66.7% (8 of 12 subjects) in the N_2O group vs. 55.6% (5 of 9 subjects) in the placebo group, with a non-significant difference between proportions of 0.11 (p = 0.67; Fisher's exact test).

C-SSRS

The Mann-Whitney *U* test showed that the score distributions were similar for both groups in all assessments. We found no significant differences in the median suicide ideation scores between groups. None of the subjects presented suicidal behavior throughout the trial (Table 2).

YMRS

There were also no significant differences in YMRS variation over time in either group (N_2O: 1.42±1.24 for 0.75±1.29; placebo: 2.44±3.00 for 1.00±2.65, $F_{15, 286}$ = 1.036, p = 0.42). The temporal distribution of mean YMRS scores is shown in Figure 3. No subject had a total YMRS score > 12 during the trial, which indicates the presence of manic episodes.

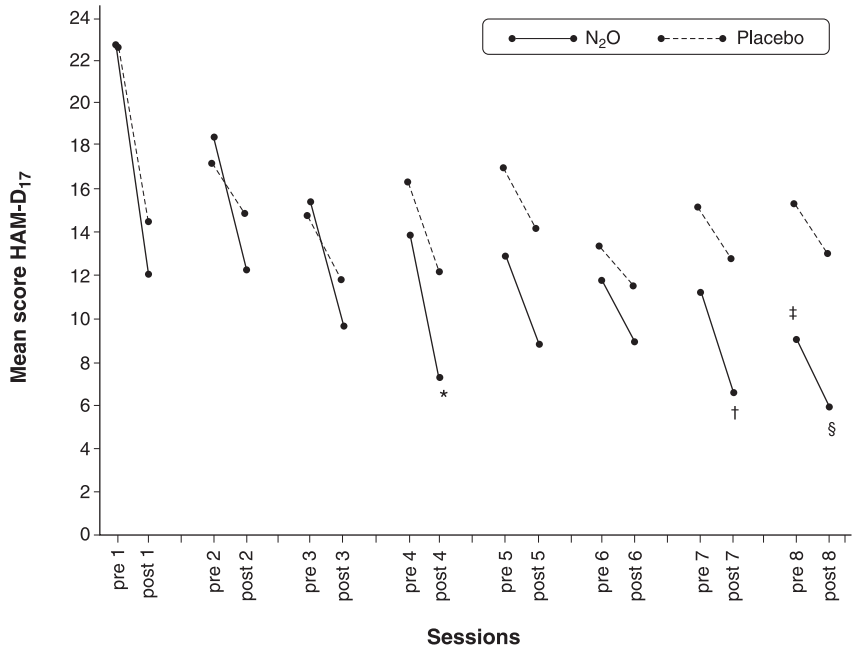

Figure 2 The effects of N_2O on depressive symptoms measured with the HAM-D_{17}. Assessment points with significant differences between groups: * session 4 post-inhalation: p = 0.027; † session 7 post-inhalation: p = 0.008; ‡ session 8 pre-inhalation: p = 0.046; § session 8 post-inhalation: p = 0.003. HAM-D_{17} = 17-item-Hamilton Depression Rating Scale; N_2O = nitrous oxide; post = post-inhalation assessment; pre = pre-inhalation assessment.

Table 2 Shift-table showing changes in Columbia-Suicide Severity Rating Scale (C-SSRS) categories from baseline during treatment (table model provided by the C-SSRS Scoring and Data Analysis Guide)[30]

	End of trial category		
Group/baseline category	No suicidal ideation or behavior, n=19 (90.0)	Suicidal ideation, n=2 (10.0)	Suicidal behavior, n=0 (0.0)
Placebo (n=9)			
No suicidal ideation or behavior	1 (11.1)	-	-
Suicidal ideation	7 (77.8)	1 (11.1)	-
Suicidal behavior	-	-	-
N_2O (n=12)			
No suicidal ideation or behavior	1 (8.3)	-	-
Suicidal ideation	10 (83.3)	1 (8.3)	-
Suicidal behavior	-	-	-

Data presented as n (%). % = 100*n/N, where: N = number of subjects in category; n = number of subjects with end of trial C-SSRS assessment.
Baseline refers to first session pre-inhalation assessment. Suicidal ideation includes any one of the five suicidal ideation events (1 = wish to be dead; 2 = non-specific active suicidal thoughts; 3 = suicidal ideation with methods but no intention to act; 4 = suicidal intention without a specific plan; and 5 = suicidal intent with plan). Each subject is counted in one cell only. Patients with both suicidal ideation and suicidal behavior were included in the suicidal behavior category.

Adverse effects

The mean inhalation time per session in the N_2O group was 59.1±4.93 minutes in a total of 96 sessions. Somnolence, paresthesia, nausea, and headache were the most frequent adverse effects. In four of the 12 subjects in this group, temporary inhalation interruptions were required in 12 different sessions for the following reasons: nausea, vomiting, emotional discomfort, and regurgitation. Five sessions were discontinued before the end, three for the same subject (at 56, 47, and 18 minutes due to nausea and regurgitation at 52 minutes, increased blood pressure at 40 minutes, and severe headache and distress soon after beginning the last inhalation session). Other adverse effects included confusion, psychomotor retardation, emotional discomfort, hearing hypersensibility, restlessness, laughter, and difficulty keeping the nasal mask on.

In the placebo group, a total of 78 sessions were performed, with a mean inhalation time per session of 59.96±0.34 minutes. Adverse effects included headache, somnolence, nausea, and difficulty keeping the nasal mask on. One subject required a temporary interruption due to nausea and severe headache, and this session was ended at 57 minutes. Regarding the monitored physiological parameters, one patient in the N_2O group had increased blood pressure in one session.

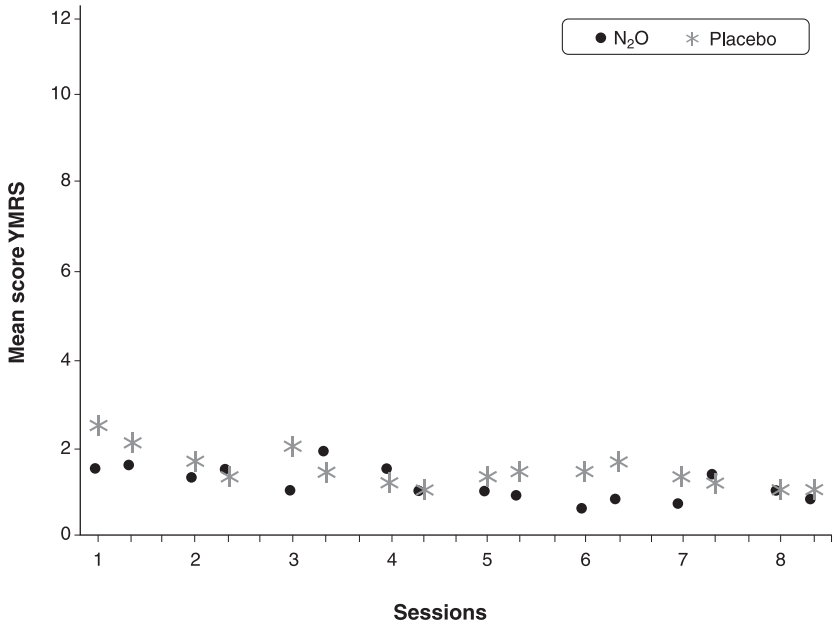

Figure 3 Time distribution of mean Young Mania Rating Scale (YMRS) score changes at each assessment point. N_2O = nitrous oxide.

Discussion

In this preliminary study, our findings show that based on HAM-D_{17} scores, the antidepressant effect of N_2O was superior to placebo. Both groups had lower HAM-D_{17} scores at the end of the trial, although the reduction was greater in the N_2O group (mean reduction of 16.66 points from baseline vs. 9.55 points in the placebo group). In addition, only the N_2O group reached a mean score within the remission range (5.92 vs. 12.89 in the placebo group). As for the placebo group, reductions in HAM-D_{17} scores vs. baseline did not fulfill the criteria for a therapeutic response. In addition to changes in mean group scores, a therapeutic response occurred in 91.7% of the N_2O group (vs. 44.4% of the placebo group), and the remission criterion was fulfilled by 75% of the N_2O group (vs. 11.1% in the placebo group). Both of these rates were significantly higher than those in the placebo group. These findings agree with the only published study on the antidepressant effects of N_2O, in which 25% of the patients in the N_2O group achieved a response (vs. 5% in the placebo group), and 15% of the patients in the N_2O group achieved remission (vs. 0% in the placebo group).[23] That study included only patients with treatment-resistant depressive disorder, which could explain the lower remission and response rates in both the active drug and placebo groups.

It is interesting to note that the antidepressant effect was both acute and cumulative. The amplitude of in-session score reduction in the N_2O group was greater than that of the placebo group in all sessions, demonstrating the acute effect of the drug, although differences in mean post-inhalation scores between the two groups were statistically significant only in the fourth, seventh, and eighth sessions ($p = 0.027$, $p = 0.008$, and $p = 0.003$, respectively). On the other hand, considering pre-inhalation assessments from the second to the eighth session as a follow-up of the effects in previous sessions, we found progressive antidepressant effects over time in the N_2O group, which demonstrates a cumulative effect, while in the placebo group this occurred only until the third session.

Unlike N_2O and ketamine, other glutamatergic modulators, such as memantine, have shown no antidepressant effects.[31] Determining which actions differentiate ketamine and N_2O from other glutamatergic modulators is important both for understanding the neurobiology of depressive disorders and for the development of accurate targets for new treatment agents. One study demonstrated that the antidepressant effect of ketamine is reversed by naltrexone, an antagonist of the opioid system.[32] Furthermore, Zarate & Machado-Vieira[33] proposed that the antidepressant effect of N_2O may result from its ability to increase concentrations of nitric oxide, a messenger that has been linked to antidepressant, anxiolytic, and analgesic effects.

Mean BDI-II scores also decreased in the N_2O and placebo groups, which indicates improvement in depressive symptoms (mean score reduction of 23.5 from baseline in the N_2O group vs. 19.97 in the placebo group). Both groups achieved mean scores within the therapeutic response range. However, changes in BDI-II scores did not reflect the same intensity of symptom improvement or the superiority of N_2O over placebo as HAM-D_{17} scores. Here, a fundamental difference between the HAM-D and BDI-II should be highlighted: the first is a clinician-rated instrument, whereas the latter is a self-rating inventory, which might result in different perceptions regarding symptom severity. Discrepancies between the perceived severity reported in these two types of instruments are frequent, and several possible explanations have been discussed in the literature. Carter et al.[34] found only a

moderate correlation between symptom severity measured with clinician-rated scales vs. self-rating scales. Nevertheless, clinician-rated scales remain the standard for studies assessing antidepressant treatments. Evidence in the literature points out a tendency for patients to perceive their symptoms as more severe than clinicians, which is in accordance with our findings.[35]

As for the improvement in depressive symptoms found in the placebo group (both through HAM-D_{17} and BDI-II scores), high response rates to placebo have been described in clinical trials of antidepressant treatments. Our placebo group showed results on par with previous findings, including BDI-II scores that did not differ significantly between groups.[36] This could lead to bias, which could result in masking or underestimation of the effects of active drugs. Among the possible explanations for the placebo effect seen in our trial, we highlight the possibility that it may derive from the care and attention received by the participants or from the self-limited nature of depressive episodes. More importantly, we cannot rule out a delayed antidepressant effect in the placebo group, since only 4 weeks without dose adjustment were an inclusion requirement.

Suicidal ideation improved in both groups by the end of the study according to the C-SSRS data, although with no significant difference between groups, unlike the improvement reported in a trial with ketamine.[37] It should be noted, however, that none of the subjects presented suicidal behavior or increased suicidal ideation during the trial.

According to the YMRS results, no manifestations of mania or hypomania were found following N_2O treatment. It is important to emphasize that a diagnosis of bipolar disorder or previous history of antidepressant-induced mania or hypomania were used as exclusion criteria in this trial.

Although we clinically evaluated the presence of dissociative and psychotomimetic symptoms in the sample, no standardized tests were used for this purpose. These effects could have been assessed through scales such as the Clinician Administered Dissociative States Scale or the Brief Psychiatric Rating Scale. We considered that since the inclusion of further instruments would have had a substantial impact on the already long trial sessions and, we opted to omit them. Nevertheless, according to clinical evaluation and subjective reports, dissociative and psychotomimetic effects were neither frequent nor intense among participants.

Regarding drug tolerability, reports of adverse effects were more frequent in the N_2O group; however, the 60-minute inhalation session could be completed in most cases without discomfort or only minimal discomfort for the subjects. In fact, subjects who received N_2O reported tranquility, relaxation, and a "sense of well-being," considering it a pleasant experience. Only one subject required temporary interruption or early discontinuation in most sessions (six of eight sessions) due to adverse effects (nausea and vomiting). Nevertheless, the subject did not consider this to be a severe discomfort and chose to remain in the trial. Among the others, six did not require interruption or early discontinuation, and the remaining five discontinued inhalation in less than half of the sessions. The main adverse effects were somnolence, nausea, vomiting, headache, and paresthesia. No serious adverse events occurred and the aforementioned effects improved rapidly after discontinuing inhalation, with no further intervention necessary, which suggests an acceptable risk-benefit ratio for the use of N_2O in patients with MDD, as was also reported by Nagele et al.[23]

Our study has certain limitations that should be pointed out. Since the trial had a small sample, our results do not represent definitive measures of efficacy and should be interpreted with caution until they are replicated in trials with larger samples. Furthermore, the trial duration was short (4 weeks) and we did not include post-treatment assessments or an intention-to-treat analysis. Of note, only two subjects abandoned the trial, both in the placebo group, one of whom reported worsening symptoms and another for personal reasons. Long-term follow-up trials are needed to establish whether the potential benefits of N_2O for depression persist over time. In addition, due to logistical issues that involved coordinating the needs of the subjects and research collaborators and the availability laboratory environment, we were unable to perform the trial sessions at the same time for all subjects. Data was not collected regarding treatment-resistant depression, but the high response rates in both groups suggest that only a few patients were treatment resistant. Finally, adverse events associated with the inhalation of N_2O may have affected the blinding procedures and revealed the use of active drugs to the subjects and researchers. With this in mind, future investigations should consider the use of active drug controls.

The most commonly used depression rating scales in clinical trials are not appropriate to detect changes in symptom severity that occur within hours or a few days. The HAM-D was developed to assess symptoms over a period of 1 week, while the BDI-II was designed to cover a period of 15 days. For example, both instruments include items related to sleep and weight that do not change rapidly. In order to circumvent this limitation, we instructed the subjects to repeat answers about sleep, appetite, and weight in scales applied within the same session, that is, before and after inhalation. A visual analogue scale or even the 6-item-Hamilton Depression Rating Scale might have been more appropriate to capture faster mood changes within the same session, which should also be kept in mind by future investigators.

Finally, up to the time of writing the initial protocol of this study, we could find no data in the literature about the best concentration of N_2O to be used in patients with depressive disorders. We chose to use a 50% concentration to avoid deep sedation and preserve participant safety in an outpatient research setting. Forthcoming studies should assess the effects of different concentrations to determine the most effective dose with the fewest adverse effects. Likewise, the frequency of N_2O administration should also be the object of future research. It is worth mentioning that administering N_2O/oxygen with portable devices is relatively easy and can be performed by non-anesthesiologists after proper training.[38] Finally, we were unable to measure the expired fraction of carbon dioxide and N_2O since the nasal masks used in the trial do

not allow coupling a capnograph or gas analyzer, which could provide important evidence about the best dose to be used.

In conclusion, this is the second trial of N_2O as a potential treatment for depression and the first to apply semiweekly doses for a month in only non-treatment-resistant patients. Further studies with larger samples, a clear distinction between treatment-resistant and non-treatment-resistant patients, and an intention-to-treat analysis are needed to determine whether N_2O is an effective antidepressant alone or as an adjuvant therapy.

Our results show that 50% N_2O administered twice a week for one month in association with antidepressant treatment in patients with persistent symptoms led to reductions in depressive symptoms as measured by the HAM-D_{17}, but not the BDI-II. In addition, 91.7% of the N_2O group achieved a therapeutic response and 75% fulfilled the criteria for remission according to the HAM-D_{17}, and 66.7% achieved response or remission according to the BDI-II. These data suggest that 50% N_2O has stronger antidepressant effects as an adjunctive treatment than antidepressants alone in patients still symptomatic after at least 4 weeks of oral pharmacotherapy.

Disclosure

The authors report no conflicts of interest.

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