INTRODUCTION

Molecular hydrogen (dihydrogen, H₂) is a novel biotherapeutic gas that appears effective in various health conditions. Hydrogen-rich water (HRW: also known as hydrogen-infused water) emerges as the most common vehicle to deliver dihydrogen in biomedicine, and over 1,500 studies published during the past decade or so confirm the favorable effects of drinking HRW in both animal and human trials (for a detailed review see Yang et al., 2020). Due to its pleiotropic biological activity and ability to easily cross the blood-brain barrier...
for 24 hr. verse events in healthy men and women who were sleep- deprived
brain metabolism, brain and oxygen saturation, and self- reported ad-
feine, and control water (no dihydrogen and caffeine) for alertness,
ing single- dose HRW, and compare it with caffeine, HRW plus caf-
aim of the present study was to evaluate the acute effects of drink-
compare HRW and caffeine for brain function. Therefore, the main
markers used in this pilot trial require additional investigation to
et al., 2020). A limited number of brain- specific outcomes and bio
orientation- specific alertness in healthy men and women (Zanini
superior to caffeine, a well- known cognitive enhancer, to improve
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Dihydrogen in HRW was produced by the following reaction: Mg +
H2O → H2 + Mg(OH)2, with magnesium used in HRW was elemen-
tal magnesium. The content of dihydrogen in all experimental drinks
was measured by gas chromatography, as previously described
(Zanini et al., 2020). All drinks were similar in appearance, texture,
and sensory characteristics, and normalized for total magnesium
amount. A wash- out period of 7 days was set to prevent the residual
effects of interventions across study periods.

Sleep deprivation was secured by keeping participants awake
in a sleep quarantine room within FSPE Applied Bioenergetics Lab
under continuous control of research staff for 24 hr before the ex-
perimental drinks’ intervention. Outcomes assessed at baseline (pre-
tervention) and 15-min follow- up were visual analogue scale (VAS)
for alertness (Srivastava et al., 2013), and the attention network test
(ANT) with subscales for alerting, orienting, and executive control
(Macleod et al., 2010). Trail- making test A was also employed as
an indicator of visual scanning, graphomotor speed, and executive
function (Llinàs- Reglà et al., 2017), along with symbol digit modal-
ities test (SDMT) (Smith, 2000). Brain oxygenation (SbO2) and he-
moglobin index (tHb) in the prefrontal cortex was monitored with
4- optode (680– 800 nm) functional near- infrared spectroscopy sen-
or (Fortiori Design LLC, Hutchinson, Minnesota), while peripheral
capillary oxygen saturation (SpO2) was monitored with fingertip
oximeter (Barrington Diagnostics, Barrington, IN). Magnetic reso-
nance imaging and spectroscopy were performed on a 1.5 T Siemens
Avanto scanner (Erlangen, Germany), using matrix head coil (receiver
coil) in circularly polarized mode as previously described (Ostojic
et al., 2017). Water- suppressed proton 2D Spectroscopic Imaging
(CSI) and single- voxel data sets were acquired with point- resolved
spectroscopy TR/TE of 1500/135 ms. The first CSI slab (field of view
(FOV) 160 × 160 × 160 mm; voxel of interest (VOI) 80 × 80 × 80 mm,

2 | METHODS

2.1 | Participants

Sixteen healthy adults (8 men and 8 women; age 24.0 ± 3.5 years,
body mass index 23.5 ± 2.4 cm) signed an informed consent to vol-
untarily participate in this randomized- controlled cross- over inter-
ventional trial. Inclusion criteria were as follows: age 18 to 30 years,
healthy body mass index (e.g., 18.5 to 25.0 kg/m²), no current chronic
diseases or acute disorders, habitual coffee drinking, and sleep
deprivation of 24 hr. Exclusion criteria included a previous history of
dietary supplement use during the four weeks before the study
commences and caffeine intake 12 hr before the trial. The minimal
sample size (n = 16) was calculated using power analysis (G*Power
3.1), with the effect's size set at 0.50 (medium effect), alpha error
probability 0.05, and power 0.80 for four groups, and eight measure-
ments of study outcomes. The primary outcome was the change in
reaction time for the attention network test (see below) at baseline
and 15- min postadministration. The study design was approved by
the local IRB at the University of Novi Sad (# 2- CFHRW/2020), with
the study systematized following the Declaration of Helsinki and
International Conference of Harmonization Efficacy Guidelines E6.

2.2 | Experimental interventions

All volunteers were allocated in a cross- over design to receive a
single- dose drink of HRW, caffeine, HRW plus caffeine, or control
drink in the morning after 24- hr sleep deprivation and 12- hr fast-
ing. The composition of each drink is presented in Figure 1, with all
experimental interventions produced by dissolving specific tablets/
powders into a cup of lukewarm water (500 ml). HRW and nonhydro-
gen producing magnesium tablets were provided by HRW Natural
Health Products Inc. (New Westminster, BC, Canada), and caffeine
anhydrous powder was purchased by Proteos (Zagreb, Croatia).

FIGURE 1 The composition of experimental interventions.
HRW—ahydrogen- rich water
2.3 | Statistical analyses

Friedman test was used to establish whether significant differences existed between different experimental drinks over time of intervention (baseline versus. 15-min postadministration), with post hoc test used to identify differences between individual sample pairs. Data were analyzed using SPSS Statistics for Mac Version 24.0 (IBM, Armonk, NY), with the significance level set at \( p < .05 \).

3 | RESULTS

All volunteers completed all four trials, with no participants reported any side effect of each intervention. Changes in alertness biomarkers were depicted in Table 1. A significant treatment versus. time interaction was found for reaction time, trail-making test duration, and SDMT outcomes \( (p < .05) \). In particular, a mean change in reaction time at 15-min follow-up was different between caffeine and HRW \( (p < .05) \), and caffeine and HRW plus caffeine \( (p < .05) \). A significantly less time was needed to complete trail-making test after both HRW and HRW plus caffeine compared with the control drink \( (p < .05) \). Finally, the number of errors in the SDMT test was significantly lower after drinking HRW or caffeine than control drink \( (p < .05) \). No differences were found for SbO2, tHb, and SpO2 during the trial \( (p > .05) \).

Figure 3 depicts changes in brain metabolic ratios across twelve different brain locations. It appears that a significant treatment versus. time interaction was found for the changes in the choline-to-creatine ratio in 9 out of 12 regions \( (p < .05) \). Caffeine significantly increased the choline-to-creatine ratio comparing to control drink in 5 locations \( (p < .05) \), HRW significantly increased the ratio in 8 locations \( (p < .05) \), and HRW plus caffeine significantly increased the ratio in 8 locations \( (p < .05) \). Besides, a combination was superior to HRW to increase the choline-to-creatine ratio in left frontal gray matter \( (p < .05) \). No differences were found between interventions for the choline-to-NAA ratio across the trial \( (p > .05) \).

4 | DISCUSSION

The present study corroborated preliminary findings that hydrogen-rich water could acutely affect biomarkers of alertness in sleep-deprived men and women. Specifically, HRW was superior to control drink to improve trail-making and SDMT performance, while caffeine outperformed HRW for reaction time. The effects seen here were accompanied by changes in brain metabolism. Both HRW and caffeine significantly increased the choline-to-creatine ratio in several brain regions (e.g., frontal white and gray matter), while HRW and the combination intervention also affected brain metabolism in the paracentral brain. These findings suggest that HRW and caffeine probably affect different domains of alertness and stimulate metabolism in separate brain segments when administered acutely in sleep-deprived volunteers.
During the past decade or so, molecular hydrogen has emerged as an innovative agent in human neuroscience and clinical neurology. The effects of this simple biomedical gas on brain performance have been evaluated in a handful of interventional trials, spanning from brain trauma to neurodegenerative and cerebrovascular diseases. For instance, hydrogen improved the brain MRI indices in the acute brain stem infarct sites (Ono et al., 2011), positively affected patient-reported scores in Parkinson’s disease (Yoritaka et al., 2013), attenuated the stroke severity in patients with cerebral infarction (Ono et al., 2017), increased mood, anxiety, and autonomic nerve function in healthy volunteers (Mizuno et al., 2018), reduced the presence and severity of symptoms of mild traumatic brain injury (Javorac et al. 2019), and improved cognitive function in community-dwelling older women (Korovljev et al., 2020). Although those studies showed promising results of medium- to long-term intervention with hydrogen on various indices of brain function, few trials endeavored to evaluate short-term effects of hydrogen on brain performance. Besides, no neuroimaging studies so far evaluated the immediate effects of hydrogen on brain metabolism in separate brain areas involved in perception, executive function, and connectivity, or contrast hydrogen with other brain-stimulating agents. Our group’s previous pilot investigation was arguably the first interventional study that compared the acute effects of single-dose HRW and caffeine on brain performance among healthy men and women who were sleep-deprived for 24 hr (Zanini et al., 2020). Both interventions acutely affected markers of alertness, yet caffeine induced a drop in alerting and executive control at 15-min follow-up, while HRW caused a reduction in the orientation at postadministration. Although no differences were found between interventions for all evaluated outcomes of alertness, the authors suggested that HRW and caffeine might have impacted different alertness domains, with dihydrogen improving orienting to sensory stimulation. In contrast, caffeine alters awareness and executive attention. The current study findings extend previous research by showing stimulating effects of acute dihydrogen and caffeine intake on brain performance and metabolism in stressed individuals, with HRW having appeared to tackle different brain regions compared with caffeine. We reported no significant differences in alertness outcomes among interventions, yet
subjectively reported scores for alertness, executive control, and test accuracy strongly tended to be improved by all three experimental interventions at follow-up, with HRW plus caffeine being the most prominent. Despite that, HRW and HRW plus caffeine were superior to control drink to complete trail-making tests in a shorter time, suggesting improved visual attention and task switching for both interventions. In addition, both caffeine and HRW were better than control drink to complete SDMT, perhaps due to intervention-driven stimulation of attention, perceptual speed, motor speed, and visual scanning reported previously (Zanini et al., 2020). Interestingly, reaction time was different for caffeine versus both HRW and HRW plus caffeine, with all interventions provoked a delay in reaction time at 15-min follow-up. To our knowledge, this is the first human study that evaluated the acute effects of dihydrogen on brain metabolism.

We found that the ameliorated brain function induced by HRW and caffeine appears to be accompanied by brain tissue metabolism adjustments. For instance, caffeine and HRW increased the choline-to-creatine ratio, an indicator of brain viability, in frontal bilateral white and gray matter, while HRW (and HRW plus caffeine) additionally elevated the ratio in the paracentral brain. This suggests that both interventions may positively affect region-specific brain metabolism linked to executive functions, attention, and problem-solving (frontal and prefrontal lobules), while HRW perhaps can also target brain regions relevant for orienting (paracentral lobule). A correlation between cognition and region-specific choline/creatine ratio has been confirmed previously (Ben Salem et al., 2008), with trail-making test results appears to be relevant not only for the frontal areas but also for more remote areas such as the thalamus, the insula, and the deep periventricular white matter. Also, we found that the combination of HRW and caffeine was equivalent or better than individual components for several alertness outcomes and brain metabolism indices. This perhaps suggests that HRW and caffeine could be consumed together while each component affects various domains of attention and brain metabolism.

Caffeine withdrawal should also be considered for study findings interpretation, with people experiencing caffeine withdrawal often experience symptoms such as headache, dizziness, fatigue, brain fog, and negative mood (Heatherley, 2011). The control group and HRW group could possibly be experiencing caffeine withdrawal symptoms while HRW might have some effect on caffeine withdrawal symptoms; future studies should recruit experimental subjects who do not habitually drink or eat caffeine or similar substances, such as theophylline. In addition, caffeine acts primarily on adenosine receptors (Costenla et al., 2010), which may cause nerve cells to speed up, since adenosine primarily causes drowsiness, and the caffeine blocks adenosine from the receptors.

Note: Values are mean ± SD.
Abbreviations: ANT, attention network test; a, control versus. HRW; b, control versus. caffeine; c, mcontrol versus. HRW plus caffeine; d, caffeine versus. HRW; e, caffeine versus. HRW plus caffeine; HRW, hydrogen-rich water. * P value from two-way mixed ANOVA (treatment versus. time interaction). Post hoc superscript indicates a significant difference at p < .05 for the following sample pairs: a, control versus. caffeine; SDMT, symbol digit modalities test; VAS, visual analogue scale.

### TABLE 1 Changes in alertness and oxygenation outcomes during the study

|                      | Baseline | Control | Caffeine | HRW     | HRW +Caffeine | P * | Post hoc |
|----------------------|----------|---------|----------|---------|--------------|-----|----------|
| VAS alertness (score)| 4.5 ± 2.6| 5.3 ± 2.2| 5.0 ± 2.4| 5.6 ± 2.3| 6.5 ± 1.9     | 0.095| -        |
| ANT alerting (ms)   | 37.0 ± 26.7| 45.6 ± 32.4| 43.6 ± 30.3| 35.3 ± 37.1| 40.3 ± 32.7 | 0.802| -        |
| ANT orienting (ms)  | 37.6 ± 27.2| 46.1 ± 28.5| 46.3 ± 22.5| 42.6 ± 25.9| 46.8 ± 25.6 | 0.963| -        |
| ANT executive control (ms) | 143.2 ± 57.7| 153.3 ± 62.7| 127.4 ± 35.1| 136.1 ± 88.6| 107.3 ± 42.2 | 0.070| -        |
| Reaction time (ms)  | 567.3 ± 81.2| 586.9 ± 76.5| 578.7 ± 71.7| 609.4 ± 68.7| 626.9 ± 90.4 | 0.041| de       |
| Test accuracy (%)   | 96.6 ± 3.4| 94.4 ± 9.9| 98.6 ± 1.3| 97.9 ± 3.1| 98.6 ± 1.4 | 0.072| -        |
| Trail-making test A (ms) | 21.6 ± 5.0| 22.8 ± 5.1| 19.3 ± 7.3| 16.6 ± 4.9| 17.0 ± 3.7 | 0.001| bc       |
| SDMT (number of symbols) | 53.2 ± 7.3| 58.6 ± 6.1| 61.2 ± 8.7| 62.9 ± 9.7| 59.1 ± 10.5 | 0.045| ab       |
| SDMT (errors)       | 1.1 ± 2.5| 1.3 ± 2.0| 0.6 ± 0.7| 0.7 ± 0.9| 1.1 ± 0.9 | 0.297| -        |
| Brain oxygenation (%)| 67.5 ± 6.0| 62.4 ± 6.6| 63.7 ± 9.0| 63.1 ± 8.1| 60.7 ± 5.8 | 0.592| -        |
| Total hemoglobin (umol/L) | 13.0 ± 0.1| 12.9 ± 0.2| 13.0 ± 0.2| 13.0 ± 0.2| 12.9 ± 0.3 | 0.803| -        |
| Peripheral oxygen saturation (%) | 98.5 ± 0.7| 98.5 ± 0.5| 98.1 ± 1.4| 98.3 ± 1.2| 98.7 ± 0.5 | 0.899| -        |
so the nerve cells tend to speed up and causes an alerting response. HRW might also interact with adenosine receptors, and this interaction should be investigated in future research. Finally, a recent study indicated that regular caffeine intake can cause minor gray matter atrophy, although it is reversible with caffeine cessation (Lin et al., 2021); this requires monitoring of structural brain changes in subsequent interventional studies with caffeine and caffeine-free beverages.

The study strengths include the use of randomized-controlled cross-over design for administering HRW, and the relatively robust methodology to evaluate both participant- and clinician-reported brain function outcomes. Our study is limited by containing a sample of young healthy subjects, so exploration on the effects of acute HRW administration in older adults or clinical populations with brain diseases is needed in order to ascertain benefits for these populations. Further studies should enroll these critical groups and perhaps evaluate the role of prolonged dihydrogen intake in experimental and clinical neurology.

5 | CONCLUSION

Drinking a single dose of hydrogen-rich water improves trail-making test performance and reduces the number of errors in symbol digit modalities test in coffee habituated sleep-deprived young adults. The attention enhancement driven by HRW appears to go with notable changes in brain metabolism, illustrated by higher choline-to-creatinine ratio levels in the frontal and paracentral brain. Being generally recognized as safe intervention (Food and Drug Administration., 2014), hydrogen could be thus recommended as a novel intervention that upholds attention in stressed conditions, with its metabolic footprint likely different from caffeine.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ETHICAL APPROVAL

The study was approved by the local IRB at the University of Novi Sad (# 2-CFHRRW/2020), with the study systematized following the Declaration of Helsinki and International Conference of Harmonization Efficacy Guidelines E6.
DATA AVAILABILITY STATEMENT
The data that support the findings of this study have already been included in the manuscript. Raw data are available from the corresponding author upon reasonable request.

REFERENCES
Bajžik, G., Auer, T., Bogner, P., Aradi, M., Kotek, G., Repa, I., Doczi, T., & Schwarcz, A. (2008). Quantitative brain proton MR spectroscopy based on measurement of the relaxation time T1 of water. *Journal of Magnetic Resonance Imaging*, 28(1), 34–38. https://doi.org/10.1002/jmri.21192

Ben Salem, D., Walker, P. M., Bejot, Y., Aho, S. L., Tavernier, B., Rouaud, O., Ricolfi, F., & Brunotte, F. (2008). N-acetylaspartate/creatine and choline/creatine ratios in the thalami, insular cortex and white matter as markers of hypertention and cognitive impairment in the elderly. *Hypertension Research*, 31(10), 1851–1857. https://doi.org/10.1291/hypres.311851

Costena, A. R., Cunha, R. A., & de Mendonça, A. (2010). Caffeine, adenosine receptors, and synaptic plasticity. *Journal of Alzheimer’s Disease*, 20(Suppl 1), S25–34. https://doi.org/10.3233/JAD-2010-91384

Food and Drug Administration. Agency Response Letter GRAS Notice No. 520. Hydrogen gas. 2014. Available at: https://www.fdanapapers.xternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=520. (Accessed at June 6, 2021)

Heatherley, S. V. (2011). Caffeine withdrawal, sleepiness, and driving performance: What does the research really tell us? *Nutritional Neuroscience*, 14(3), 89–95. https://doi.org/10.1080/147683011X13019262348785

Iketani, M., & Ohsawa, I. (2017). Molecular Hydrogen as a Neuroprotective Agent. *Current Neuropharmacology*, 15(2), 324–331. https://doi.org/10.2174/1570159x14666160607205417

Javorac, D., Stajer, V., & Ostojic, S. M. (2019). Case Report: Buccal ad

Korovljev, D., Trivic, T., Stajer, V., Drid, P., Sato, B., & Ostojic, S. M. (2020). Short-term H₂ inhalation improves cognitive function in older women: A pilot study. *International Journal of Gerontology*, 14(2), 149–150. https://doi.org/10.6890/IJGE.202005_14(2).0013

Lin, Y. S., Weibel, J., Landolt, H. P., Santini, F., Meyer, M., Brunmair, J., Meier-Menches, S. M., Gerer, C., Borgwardt, S., Cajochen, C., & Reichert, C. (2021). Daily caffeine intake induces concentration-dependent medial temporal plasticity in humans: A multimodal double-blind randomized controlled trial. *Cerebral Cortex*, 31(6), 3096–3106. https://doi.org/10.1093/cercor/hbab005

Llinás-Reglá, J., Vilalta-Franch, J., López-Pousa, S., Calvó-Persax, L., Torrents Rodas, D., & Garre-Olmo, J. (2017). The Trail Making Test. *Assessment*, 24(2), 183–196. https://doi.org/10.1177/1073191115602552

Macleod, J. W., Lawrence, M. A., McConnell, M. M., Eskes, G. A., Klein, R. M., & Shore, D. I. (2010). Appraising the ANT: Psychometric and theoretical considerations of the Attention Network Test. *Neuropsychology*, 24(5), 637–651. https://doi.org/10.1037/a0019803

Mizuno, K., Sasaki, A. T., Ebisu, K., Tajima, K., Kajimoto, O., Nojima, J., Kuratsune, H., Hori, H., & Watanabe, Y. (2018). Hydrogen-rich water for improvements of mood, anxiety, and autonomic nerve function in daily life. *Medical Gas Research*, 7(4), 247–255. https://doi.org/10.4103/2045-9912.222448

Ohta, S. (2012). Molecular hydrogen is a novel antioxidant to efficently reduce oxidative stress with potential for the improvement of mitochondrial diseases. *Biochimica et Biophysica Acta (BBA) - General Subjects*, 1820(5), 586–594. https://doi.org/10.1016/j.bbagen.2011.05.006

Ono, H., Nishijima, Y., Adachi, N., Tachibana, S., Chitoku, S., Mukaihara, S., Sakamoto, M., Kudo, Y., Nakazawa, J., Kaneko, K., & Nawashiro, H. (2011). Improved brain MRI indices in the acute brain stem infarct sites treated with hydrosyl radical scavengers, Edaravone and hydrogen, as compared to Edaravone alone. A non-controlled Study. *Medical Gas Research*, 1(1), 12. https://doi.org/10.1186/2045-9912-1-12

Ono, H., Nishijima, Y., Ohta, S., Sakamoto, M., Kinone, K., Horikosi, T., Tamaki, M., Takeshita, H., Futatuki, T., Ohishi, W., Ishiguro, T., Okamoto, S., Ishii, S., & Takeshima, H. (2017). Hydrogen Gas Inhalation Treatment in Acute Cerebral Infarction: A Randomized Controlled Clinical Study on Safety and Neuroprotection. *Journal of Stroke and Cerebrovascular Diseases: the Official Journal of National Stroke Association*, 26(11), 2587–2594. https://doi.org/10.1016/j.jstrokecerebrovasdis.2017.06.012

Ostojic, S. M. (2017). Does H₂ Alter Mitochondrial Bioenergetics via GHS-R1a Activation? *Theranostics*, 7(5), 1330–1332. https://doi.org/10.7150/thno.18745

Ostojic, S. M., Ostojic, J., Drid, P., Vranes, M., & Jovanov, P. (2017). Dietary guanidinoacetic acid increases brain creatine levels in healthy men. *Nutrition*, 33, 149–156. https://doi.org/10.1016/j.nut.2016.06.001

Smith, A. (2000). *Symbol Digit Modality Tests*: SDMT. Testzentrale.

Srivastava, S., Donaldson, L. F., Rai, D., Melichar, J. K., & Potokar, J. (2013). Single bright light exposure decreases sweet taste threshold in healthy volunteers. *Journal of Psychopharmacology*, 27(10), 921–929. https://doi.org/10.1177/0269881113499206

Vanhamme, L., van den Boogaart, A., & Van Huffel, S. (1997). Improved method for accurate and efficient quantification of MRS data with use of prior knowledge. *Journal of Magnetic Resonance*, 129(1), 35–43. https://doi.org/10.1006/jmre.1997.1244

Yang, M., Dong, Y., He, Q., Zhu, P., Zhuang, Q., Shen, J., Zhang, X., & Zhao, M. (2020). Hydrogen: A Novel Option in Human Disease Treatment. *Oxidative Medicine and Cellular Longevity*, 2020, 8384742. https://doi.org/10.1155/2020/8384742

Yoritaka, A., Takanashi, M., Hirayama, M., Nakahara, T., Ohta, S., & Hattori, N. (2013). Pilot study of H₂ therapy in Parkinson’s disease: A randomized double-blind placebo-controlled trial. *Movement Disorders*, 28(6), 836–839. https://doi.org/10.1002/mds.25375

Zanini, D., Stajer, V., & Ostojic, S. M. (2020). Hydrogen vs. caffeine for improved alertness in sleep-deprived humans. *Neuropsychology*, 52(1), 67–72. https://doi.org/10.1007/s10880-020-09852-7

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