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# Hyperpolarized [1-$^{13}$C]pyruvate Magnetic Resonance Spectroscopy Shows That Agmatine Increased Lactate Production in the Brain of Type 2 Diabetic Mice

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**Purpose:** Type 2 diabetes mellitus (T2DM) is associated with a 2-fold increased risk of developing Alzheimer's disease. In earlier research, agmatine has been demonstrated to alleviate diabetes symptoms and increase cognitive performance. However, it is unclear whether the improvement of cognitive function is attributable to the reduction of diabetic symptoms or its direct influence on brain metabolism. Using hyperpolarized (HP) [1-$^{13}$C]pyruvate magnetic resonance spectroscopy (MRS), this study intends to evaluate the influence of agmatine on brain metabolism.

**Materials and Methods:** ICR mice were fed a high-fat diet and injected with streptozotocin to develop a T2DM animal model. During a 2-week period, T2DM mice were treated with normal saline or 100 mg/kg of agmatine, and brain HP [1-$^{13}$C]pyruvate MRS was performed. The effect of agmatine on lactate generation and NADH/NAD+ redox state was investigated using C6 and neuro-2a (N2a) cells.

**Results:** As a perfusion marker, the total $^{13}$C signals in the brain of T2DM mice ($p$=0.07) and agmatine-treated mice ($p$<0.05) were reduced. The conversion constant ($K_{pl}$) from [1-$^{13}$C]pyruvate to [1-$^{13}$C]lactate was not distinguishable in the brains of T2DM mice but was significantly increased in the brains of agmatine-treated T2DM mice. Treating C6 and N2a cells with agmatine increased NADH/NAD+ ratio and lactate generation.

**Conclusion:** Agmatine influences the NADH/NAD+ redox state in the brains of T2DM mice, which may be connected with enhanced cognitive performance and increased conversion of HP [1-$^{13}$C]pyruvate to HP [1-$^{13}$C]lactate.

**Key Words:** Alzheimer's disease, hyperpolarized $^{13}$C, magnetic resonance spectroscopy, agmatine, lactate

## INTRODUCTION

The involvement of metabolic abnormalities in the onset of dementia is the subject of a growing body of research. An epidemiological study reported the frequency of dementia in patients among diabetic patients.[1] Since type 2 diabetes mellitus

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The research paper entitled "Hyperpolarized [1-$^{13}$C]pyruvate magnetic resonance spectroscopy indicates that agmatine augments lactate production in the brain of type 2 diabetic mice" was presented as a poster by Ho-Taek Song during the European Molecular Imaging Meeting in 2023, held from March 12th to 15th.
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(T2DM) has a long-term development and the factors impacting metabolisms, such as blood glucose, insulin, and glucagon, alter dynamically,[2] it is difficult to determine the diabetes-related factors that induce cognitive deterioration. Therefore, technologies to monitor brain metabolism during long-term disease progression are required to determine the cause and diagnosis of dementia caused by diabetes. Fluorine 18 ($^{18}$F) fluorodeoxyglucose (FDG) positron emission tomography (PET) is a common metabolic imaging technique used to detect glucose uptake. Reduced $^{18}$F FDG uptake in the brain is associated with reduced glucose transporter (GLUT1 and GLUT3) expression in the brains of Alzheimer's disease patients.[3-5] However, frequent use of radioactive isotopes during follow-up has limitations. A serum glucose level of more than 160 mg/dL can alter the $^{18}$F FDG standardized uptake value, limiting its application in diabetic patients.[6,7]

Without exposure to ionizing irradiation,[8] hyperpolarized (HP) $^{13}$C pyruvate magnetic resonance spectroscopy (MRS) can trace metabolism with roughly 10000-fold increased sensitivity. It traces the conversion of injected [1-$^{13}$C]pyruvate to [1-$^{13}$C]lactate mediated by the enzyme. Consequently, it is comparatively unaffected by serum glucose levels.[9] Prior to this study, we used HP $^{13}$C MRS to trace regional brain metabolism in pre-diabetic mice fed a 60% high-fat diet (HFD) and in T2DM injected with a single dose of streptozotocin and fed a 60% HFD. In pre-diabetes, the [1-$^{13}$C]lactate/[1-$^{13}$C]pyruvate ratio increased around 4–5 folds in the entire brain, the greatest rise occurring in the hippocampus region, whereas a 1.4-fold increase was detected in the hippocampal region in the T2DM group.[10,11] These findings suggest that the brain response to metabolic state varies with illness progression.

Anti-diabetic medicines, such as dipeptidyl Peptidase-4 inhibitors, metformin, thiazolidinedione, and sulfonylurea, demonstrated a beneficial therapeutic effect on dementia associated with diabetes.[12,13] Agmatine is an endogenous aminoguanidine, and its therapeutic effects in diabetes, stroke, spinal cord injury, and cognitive decline have been reported.[14,15] We recommended the use of agmatine as a therapeutic option for diabetes-induced dementia. In a previous study,[14] we demonstrated that agmatine prevented T2DM-mediated cognitive decline in an animal model. A nearly 4-fold increase in glucose tolerance, insulin tolerance, and overnight fasting blood glucose levels was found in T2DM, and treatment with agmatine for 2 weeks returned the levels to those of control mice. Insulin levels in mice with T2DM increased nearly 10-fold, but agmatine decreased them by 4.5-fold. T2DM mice had a cognitive impairment on behavior tests, whereas agmatine therapy increased their performance on the Morris water maze test and Nesting building task. This enhanced cognition was supported by decreased tau phosphorylation and amyloid beta buildup in the brain cortex and hippocampus.[14] However, it is unknown if agamatine's effect on cognitive enhancement resulted from the alleviated diabetic symptoms or whether it directly affected brain metabolism. Using HP [1-$^{13}$C]pyruvate MRS, this study evaluated the influence of agmatine on brain metabolism in mice with T2DM.

## MATERIALS AND METHODS

### Animal procedures

We previously documented the effect of agmatine on a T2DM model with Alzheimer's-like changes; therefore, we examined HP $^{13}$C pyruvate MRS in the same animal model. Seven-week-old adult male ICR mice were used (Central Lab Animal Inc., Seoul, Korea). All procedures were carried out in compliance with the standards of the Yonsei University College of Medicine Animal Care and Use Committee and the National Institutes of Health. Normal control mice (n=4) were fed normal chow for 14 weeks. To induce T2DM, eight mice were fed a HFD (60 kcal fat) for 1 month, injected intraperitoneally with streptozotocin (100 mg/kg/ip) dissolved in citrate buffer (pH 4.4), and then fed a HFD for another 2 months. After dividing the mice into two groups, the vehicle (HFD) group was intraperitoneally injected with saline (n=4), and the agmatine (HFD+Agm) group was injected with 100 mg/kg/ip of agmatine (Sigma Aldrich, St. Louis, MI, USA) dissolved in saline for 2 weeks (n=4). At 14 weeks, HP $^{13}$C pyruvate MRS was done.

### HP $^{13}$C MR spectroscopy

Mice fasted for 4 to 5 hours before the HP $^{13}$C MR spectroscopy. We mixed 26.7 mg of [1-$^{13}$C]pyruvic acid (Cambridge Isotope, Tewksbury, MA, USA) with 15 mM of trityl radical OX-063 (Oxford Instruments, Oxford, UK) and 0.75 mM of gadoterate meglumine (Dotarem®; Guerbet, Villepinte, France). The [1-$^{13}$C]pyruvic acid sample was HP using a dynamic nuclear polarization device (HyperSense®; Oxford Instruments) and dissolved in 3.8 mL of Tris/EDTA-NaOH buffer, yielding 79 mM pyruvate (pH7.5) with an approximately 20% polarization level. For in vivo MRS, 350 μL of HP [1-$^{13}$C]pyruvate was drawn into a syringe. Using a 9.4T animal imaging system (BioSpec 94/20, Bruker BioSpin MRI GmbH, Ettlingen, Germany) with a $^{1}$H-$^{13}$C dual-tuned surface transmit/receive coil, we performed in vivo HP MR spectroscopy. We obtained time-resolved $^{13}$C free induction decay data from 7.5 mm axial slices at the mid-level of the brain containing hippocampus using a pulse-and-acquire sequence[16] with a flip angle of 10° and a temporal resolution of 1 second. The other parameters were the field of view=24×24 mm$^2$, matrix size=12×12, echo time (TE)/repetition time (TR)=26.8/81 ms. As a marker of perfusion, we computed the sum of the area beneath the $^{13}$C spectrum for 10 seconds after injection. The pyruvate to lactate conversion rate constant ($k_{pl}$) was determined by fitting the lactate and pyruvate peak intensities to the two-site exchange model presented by Day:[17]


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$$dL/dt=-\rho_l (L-L_\infty)+kp\ P-kl\ L \quad \text{(eq.1)}$$
$$dP/dt=-\rho_p (P-P_\infty+kp\ L-kp\ P \quad \text{(eq.2)}$$

where L and P are the z-magnetizations of $^{13}C$ nucleus in the lactate and pyruvate; t is time; $\rho_l$ and $\rho_p$ are the spin-lattice relaxation rates for lactate and pyruvate; $L_\infty$ and $P_\infty$ are the equilibrium magnetizations of lactate and pyruvate; kp is the conversion rate constant from pyruvate to lactate; and kl is the conversion rate constant from lactate to pyruvate. The spin-lattice relaxation rates for lactate and pyruvate were assumed to be the same. The equilibrium magnetizations are assumed by the concentrations of lactate and pyruvate. All data were processed using MATLAB-based analysis (R2017a; MathWorks, Natick, MA, USA).

### Cell culture

C6 glioblastoma cells and Neuro-2a (N2a) cells were grown in T75 cell culture flasks (SPL Life Science, Pocheon, Korea) with 10% fetal bovine serum (FBS, Corning, Corning, NY, USA) containing high-glucose Dulbecco's modified Eagle's medium (DMEM, Hyclone, Logan, UT, USA). The cells were incubated with 5% $CO_2$ at 37°C. To examine the effect of agmatine on the NADH/NAD+ ratio or amount of lactate, cells were incubated in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% FBS since DMEM contained 110 mg/L of sodium pyruvate (Sigma Aldrich), which can affect the NADH/NAD+ ratio and lactate level.[18]

### Assessment of nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide hydrate (NADH)

C6 and N2a cells were grown at a density of $1\times10^6$ cells per 6 cm dish using 10% FBS-containing DMEM. After 24 hours, the cells were treated with 2.5 or 5 mM agmatine in RPMI 1640 media containing 10% FBS. NAD and NADH levels were measured using the NADH/NAD+ Colorimetric kit (K337, Biovision, Milpitas, CA, USA). Cells that have been trypsinized were collected and lysed using NADH/NAD+ extraction buffer according to the manufacturer's instructions. We quantified the concentrations of NAD+ and NADH using Bicinchoninic Acid (BCA)-based analysis with the same amount of solute. The absorbance was measured at 450 nm using a Versamax spectrometer (Molecular Device, San Jose, CA, USA).

### Assessment of lactate level

C6 and N2a cells were seeded in a density of $1\times10^6$ cells per 6 cm dish with 10% FBS-containing DMEM. After 24 hours, the cells were treated with 2.5 or 5 mM agmatine in RPMI 1640 medium (Hyclone) containing 10% FBS with or without 110 mg/L sodium pyruvate. Cells were lysed using lactate assay buffer contained in the lactate colorimetric assay kit (K607, Biovision). The concentration of the lysate was determined using a BCA protein kit (Thermo Scientific, Waltham, MA, USA). According to the manufacturer's instructions, the lactate concentration was measured and computed in 40 μg of lysate.

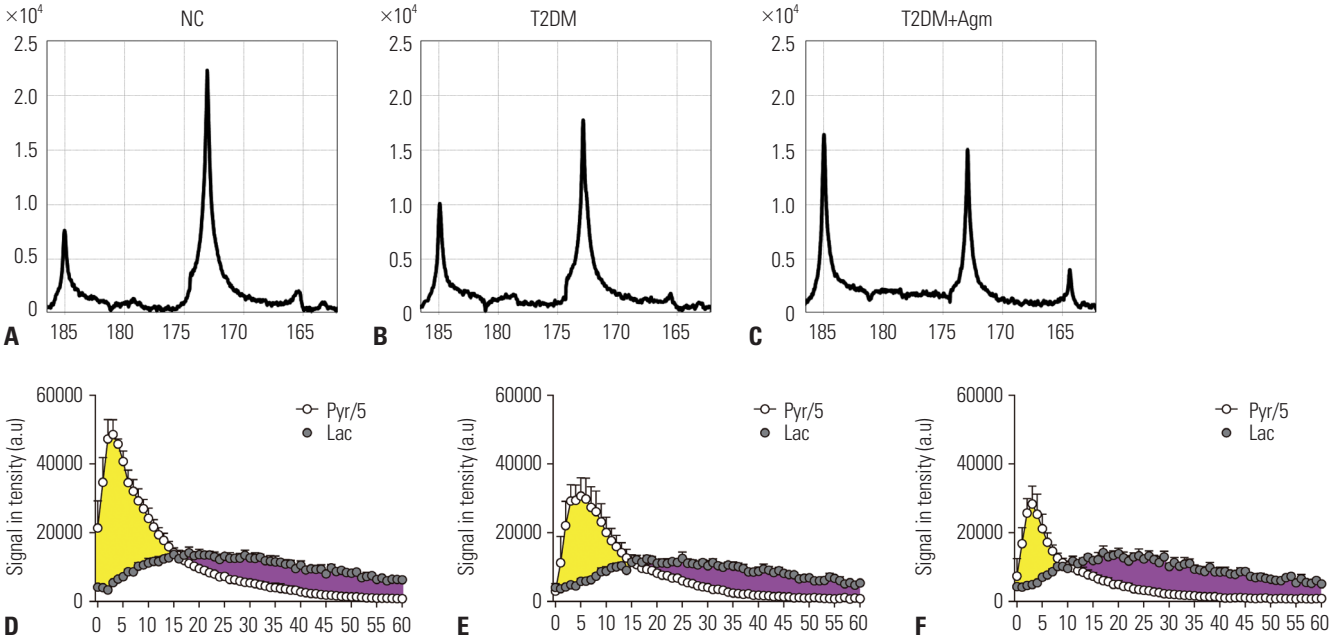


**Fig. 1.** HP $^{13}C$ lactate peak intensity was increased in the brains of T2DM mice treated with agmatine. (A) The representative sum of the $^{13}C$ spectrum was measured in the brains of normal control mice, (B) T2DM mice, and (C) T2DM mice treated with agmatine. In the complete brains of mice, [1-$^{13}C$] pyruvate, [1-$^{13}C$]lactate, and $^{13}C$ bicarbonate was identified at 173 ppm, 185 ppm, and 162 ppm, respectively. (D) Dynamic peak intensity of [1-$^{13}C$]pyruvate and [1-$^{13}C$]lactate in the brain of (D) normal control mice, (E) T2DM mice, and (F) agmatine-treated T2DM mice (n=4 mice). Graphs from injection to 60 seconds are depicted using an arbitrary unit (a.u.). The regions that contain more pyruvic acid than lactate are colored yellow, whereas the areas with more lactate than pyruvic acid are colored purple. HP, hyperpolarized; T2DM, type 2 diabetes mellitus; NC, normal control.

### Statistical analysis

The in vivo data are expressed as mean±standard error, while in vitro data are expressed as mean±standard deviation. The sample size reflects the experimental replications from a single representative experiment validated by independent repetitions. $p<0.05$ was considered statistically significant. $p$-values were determined by analysis of variance followed by Tukey's test for post-hoc comparison using statistical software (PRISM, version 6.0, GraphPad Software, San Diego, CA, USA).

## RESULTS

### HP $^{13}$C lactate peak increased in the brain of agmatine-treated T2DM mice

We obtained the spectra (Fig. 1A-C) and dynamic curves (Fig. 1D-F). In the brains of mice, [1-$^{13}$C]pyruvate was found at 173 ppm, [1-$^{13}$C]lactate at 185 ppm, and $^{13}$C bicarbonate at 162 ppm. The brain of the agmatine-treated T2DM mice showed the greatest $^{13}$C lactate peak (Fig. 1A-C), and greater lactate conversion was found in the dynamic curve of the agmatine-treated group (Fig. 1D-F).

### Agmatine-treated T2DM mice did not affect brain perfusion but increased lactate conversion

As agmatine improved a number of blood markers in T2DM,[14] we investigated whether it also improved brain perfusion. Hypoperfusion was seen in the brains of mice with T2DM, as expected ($p$=0.074). Still, agmatine did not alleviate cerebral hypoperfusion ($p$=0.020) (Table 1, Fig. 2A). The value of the conversion rate constant from pyruvate to lactate ($K_{pl}$) did not change between T2DM and control groups ($p$=0.255). Still, it was considerably increased in the agmatine-treated T2DM group ($p$=0.012), indicating that agmatine promotes lactate conversion in the brains of T2DM mice (Table 1, Fig. 2B). However, the ratio of HP $^{13}$C bicarbonate/pyruvate did not differ between the groups (Fig. 2C).

### Agmatine increased NADH/NAD+ ratio and lactate production in both neuron and astrocyte in vitro

Metformin is known to alter the cytosolic NADH/NAD+ redox state,[19,20] resulting in increased cytosolic NADH levels, which can increase $K_{pl}$ by utilizing NADH.[21] Given that agmatine has a similar guanidine structure to metformin and a similar effect on serum glucose levels in diabetic mice,[14] we examined whether agmatine might affect the NADH/NAD+ redox state and the amount of lactate.

As demonstrated in Fig. 3, agmatine administration raised the amount of NADH and the NADH/NAD+ ratio in both neuronal N2a cells and astroglial C6 cells. In addition to an increase in the NADH/NAD+ ratio, agmatine administration elevated lactate levels, which were further increased by the addition of sodium pyruvate (Fig. 4). These findings suggest that agmatine affects the ratio of NADH to NAD+ in the T2DM brain. Therefore, when HP [1-$^{13}$C]pyruvate was supplied, it was forced to be converted to [1-$^{13}$C]lactate by the consumption of NADH.

## DISCUSSION

Agmatine has been found to relieve diabetes symptoms and enhance cognitive function.[14] In this study, HP [1-$^{13}$C]pyruvate MRS was used to explore the effect of agmatine therapy on brain metabolism in a T2DM model.[11] As a perfusion marker, the total HP $^{13}$C signals in the brain of mice were reduced in T2DM, and agmatine-treated mice showed no perfusion improvement. The [1-$^{13}$C]pyruvate to [1-$^{13}$C]lactate conversion

**Table 1.** The sum of $^{13}$C spectrum and $K_{pl}$

| | NC | T2DM | T2DM+Agm |
|---|---|---|---|
| Sum of $^{13}$C spectrum (arbitrary unit) | $4.6\times10^7\pm3.7\times10^6$ | $3.4\times10^7\pm3.7\times10^6$ | $3.0\times10^7\pm2.7\times10^6$ |
| $K_{pl}$ | 0.0087±0.0011 | 0.0114±0.0014 | 0.0173±0.0008 |

NC, normal control; T2DM, type 2 diabetes mellitus.
$K_{pl}$: Apparent rate constant for conversion of hyperpolarized [1-$^{13}$C]pyruvate to [1-$^{13}$C]lactate.

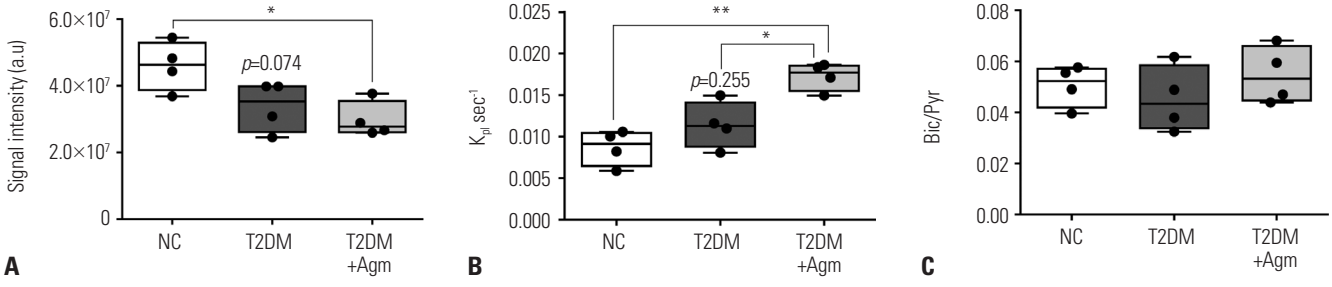


**Fig. 2.** Treatment with agmatine had no effect on cerebral brain perfusion, although it increased [1-$^{13}$C]lactate/pyruvate conversion. (A) The box plot depicts the total $^{13}$C signal collected 10 seconds after the injection as a perfusion indicator from the brain. (B) Apparent conversion rate constant from HP [1-$^{13}$C]pyruvate to HP [1-$^{13}$C ]lactate ($K_{pl}$). (C) The peak bicarbonate-to-pyruvate (Bic/Pyr) signal ratio was computed. $p$-value was obtained from ANOVA, followed by the Tukey test for post-hoc comparisons. *$p$<0.05; **$p$<0.01. HP, hyperpolarized; ANOVA, analysis of variance.


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rate constant ($K_{pl}$) did not differ between the control and T2DM groups. However, it nearly doubled when treated with agmatine, indicating a greater lactate concentration. As agmatine treatment of N2a and C6 cells increased the NADH/NAD+ ratio and increased the amount of lactate, our data showed that agmatine in the brain altered the cytosolic NADH/NAD+

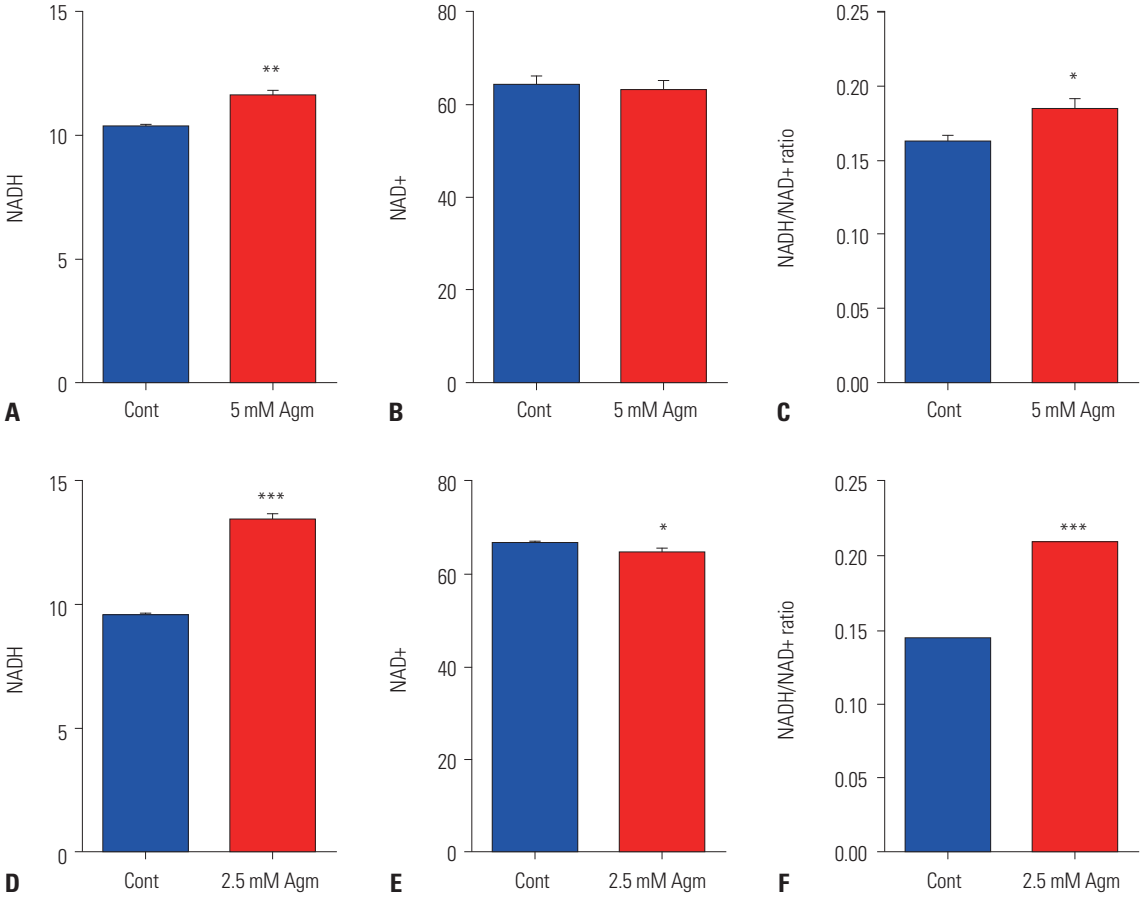


**Fig. 3.** Agmatine raises the ratio of NADH to NAD+ in C6 cells and N2a cells. (A) NADH, (B) NAD+, and (C) NADH/NAD+ ratio was determined in glial C6 cells 24 hours after treatment with 5 mM agmatine. (D) NADH, (E) NAD+, and (F) the ratio of NADH to NAD+ in neuronal N2a treated with 2.5 mM agmatine. *p*-value was obtained from ANOVA, followed by the Tukey test for post-hoc comparisons. **p*<0.05; ***p*<0.01; ****p*<0.001. N2a, neuro-2a; ANOVA, analysis of variance; RPMI, Roswell Park Memorial Institute.

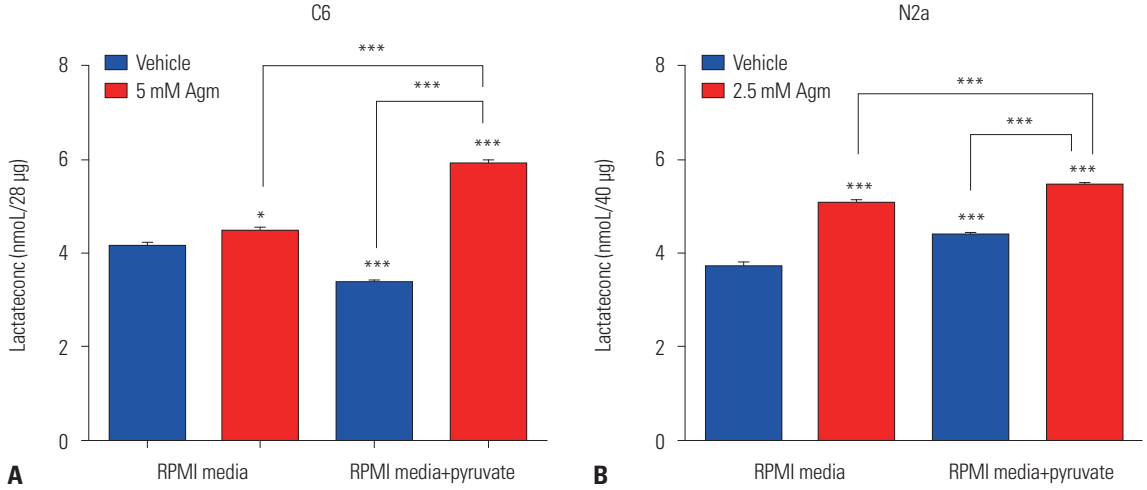


**Fig. 4.** Agmatine increases lactate production in C6 cells and N2a cells. Lactate concentration was measured 24 hours after treating (A) glial C6 cells with 5 mM agmatine without or with 110 mg/L of sodium pyruvate and (B) neuronal N2a cells with 2.5 mM agmatine without or with 10 mg/L of sodium pyruvate. *p*-value was obtained from ANOVA, followed by the Tukey test for post-hoc comparisons. **p*<0.05; ****p*<0.001. N2a, neuro-2a; ANOVA, analysis of variance; RPMI, Roswell Park Memorial Institute.

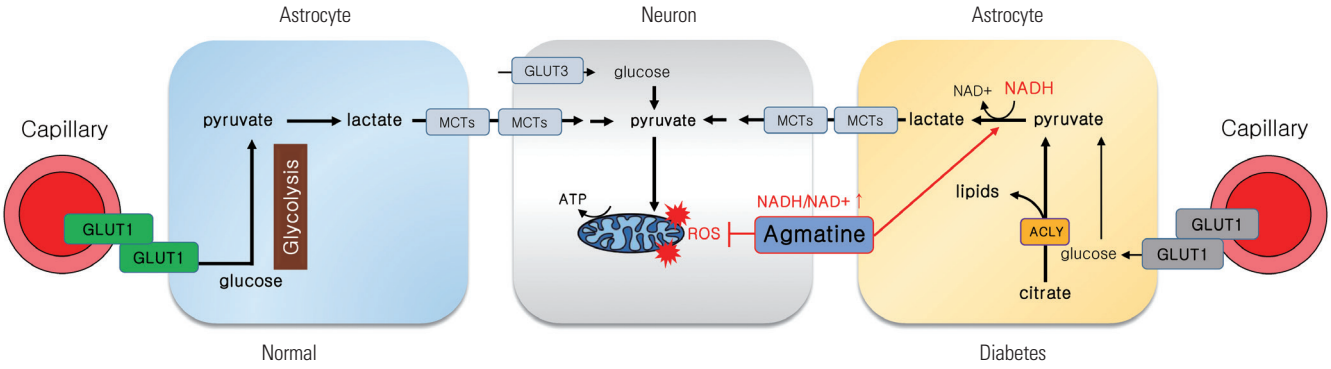


**Fig. 5.** Function of agmatine on the astrocyte-neuron lactate shuttle in the T2DM brain. Glucose is mostly delivered to astrocytes, metabolized to lactate, and released through monocyte transporters (MCTs) in the normal brain. Through mitochondrial metabolism, neurons absorb lactate, convert it to pyruvate, and produce ATP. T2DM leads to decreased glucose availability in the brain due to insulin resistance or decreased glucose transporter1 (GLUT1). As agmatine influences cytosolic NADH/NAD+ redox states, decreased ROS production in neurons and increased lactate production in astrocytes may be associated with enhanced cognitive function in T2DM. T2DM, type 2 diabetes mellitus; ATP, adenosine triphosphate; ROS, reactive oxygen species.

redox state in the brain of T2DM, which may contribute to enhanced cognitive performance.

Endogenous agmatine's physiological function is mainly unclear. However, exogenous agmatine has shown neuroprotective benefits in various neurodegenerative illnesses, such as Alzheimer's disease, Parkinson's disease, and brain ischemia.[15] Increased reactive oxygen species (ROS) levels significantly impact neuronal cell death. Previously, it was discovered that neuronal cell exposure to high glucose-induced ROS formation and apoptosis, but agmatine decreased this high glucose-mediated ROS production and apoptosis.[22] When the glucose supply is excessive, NADH production through glycolysis and the tricarboxylic acid cycle increase, promoting ROS production in the electron transport chain in mitochondria to generate adenosine triphosphate (ATP).[23] Increasing cytosolic NADH by agmatine in neuronal cells can inhibit ROS generation in the presence of excessive energy load or defective mitochondria. Restricting pyruvate entry into mitochondria can minimize ROS production by increasing lactate production using NADH in the cytoplasm. This is because ROS are formed largely in the mitochondria (Fig. 5).

Recently, the astrocyte-neuron lactate shuttle hypothesis has highlighted lactate's significance, primarily produced by astrocytes and utilized by neurons for ATP production.[24,25] Since astrocytes have greater metabolic flexibility than neurons under stress conditions, such as hypoxia or diabetes, lactate production in astrocytes can aid in maintaining neuronal function by being converted to pyruvate and utilized in mitochondrial metabolism to make ATP. In a previous study, we hypothesized that the ATP citrate lyase-mediated metabolic route promoted lactate production in the brain of T2DM in response to decreased glucose availability. Despite the fact that agmatine boosts lactate production in astrocytes by regulating the NADH/NAD+ ratio, it may benefit the brain by avoiding the ATP citrate lyase-mediated metabolic route, which can stimulate lipid buildup in the brain (Fig. 5). However, it has to be determined whether agmatine-mediated lactate production can affect the ATP citrate lyase-mediated metabolic pathway and lipid accumulation in astrocytes.

Interestingly, despite the fact that agmatine therapy did not improve brain perfusion, it had a direct effect on brain metabolism. This is noteworthy as alleviating symptoms of T2DM is one strategy to reduce the incidence of dementia. Metformin is an intriguing medicine in this way since its guanidine structures resemble those of agmatine. However, the results of metformin use in Alzheimer's disease have been varied. According to research, metformin decreases insulin resistance and oxidative stress in the brain, improving cognitive function.[26,27] On the other hand, it has been hypothesized that long-term use of metformin in T2DM is a risk factor for neurodegenerative diseases.[28] A recent study found that metformin had no effect on patients with less than 5 years of T2DM duration, while the risk of developing Alzheimer's disease increased with longer diabetes duration (>5 years).[29] The mixed results of metformin on cognitive function may be due to the fact that metformin has a neuroprotective effect on the brain of T2DM, which can be offset by negative effects with extended administration. It is important to remember that metformin affects ATP generation, and metformin's potential to impair brain function may result from its ongoing ATP depletion.

The significance of guanidine-containing medication in modulating the NADH/NAD+ ratio, which may be their mechanism of action, is highlighted by our research. In addition, when utilizing HP [1-$^{13}$C]pyruvate MRS to evaluate therapeutic efficacy in certain disorders, it is essential to determine whether the medicine comprises a guanidine structure. In a recent study, we found that ionizing radiation therapy decreased HP [1-$^{13}$C] lactate conversion in an animal model breast cancer brain metastasis, indicating a decrease in the Warburg effect. Metformin's influence on the NADH/NAD+ ratio rendered HP [1-$^{13}$C] pyruvate useless for evaluating the combined effect when ionizing radiation therapy was paired with metformin.

A limitation of our study is that we did not investigate the locoregional effects of agmatine on brain metabolism using


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multivoxel mapping. In our previous research, we used chemical shift imaging mapping to indicate locoregional variations in brain metabolism in T2DM mice, namely that HP [1-$^{13}$C]lactate production was considerably elevated in the hippocampus region. In the present work, as a probing study, we obtained HP $^{13}$C spectra using time-resolved single-voxel MRS at the mid-level of the brain containing the hippocampus to investigate whether agmatine affects brain metabolism, including the hippocampus. Further investigation using multivoxel MRS mapping would be valuable to evaluate the locoregional variation of brain response by agmatine treatment.

In conclusion, agmatine influences brain metabolism in T2DM by altering the ratio of NADH to NAD+, resulting in an increase in HP [1-$^{13}$C]lactate conversion but no improvement in hypoperfusion. This suggests that medication altering the ratio of NADH to NAD+, such as metformin and agmatine, may be helpful for T2DM-mediated cognitive decline, and that prior knowledge of a drug's effect on the NADH/NAD+ redox state is essential.

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## AUTHOR CONTRIBUTIONS

Conceptualization: Eosu Kim, Chul Hoon Kim, Jong Eun Lee, and Ho-Taek Song. Data curation: Young-Suk Choi, Jae Eun Song, and Ho-Taek Song. Formal analysis: Young-Suk Choi and Jae Eun Song. Funding acquisition: Eosu Kim, Chul Hoon Kim, Jong Eun Lee, and Ho-Taek Song. Investigation: Young-Suk Choi, Eosu Kim, Chul Hoon Kim, Jong Eun Lee, and Ho-Taek Song. Methodology: Young-Suk Choi, Jong Eun Lee, and Ho-Taek Song. Project administration: Jong Eun Lee and Ho-Taek Song. Resources: Jong Eun Lee and Ho-Taek Song. Software: Jae Eun Song. Supervision: Jong Eun Lee and Ho-Taek Song. Validation: Jong Eun Lee and Ho-Taek Song. Visualization: Jae Eun Song. Writing—original draft: Young-Suk Choi and Ho-Taek Song. Writing—review & editing: Young-Suk Choi, Jae Eun Song, and Ho-Taek Song. Approval of final manuscript: all authors.

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