Title page

(1) Full title

Antioxidative effects of a novel dietary supplement Neumentix in a mouse stroke model

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(6) Shortened title

Antioxidative effects of Neumentix in a stroke model

(7) Keywords
ROS, infarction volume, mint, rosmarinic acid

(Abbreviations used)
CML, Nε-(carboxymethyl) lysine; 4-HNE, 4-hydroxy-2-nonenal; MCA, middle cerebral artery; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; PBS, phosphate-buffered saline; RA, rosmarinic acid; tMCAO, transient middle cerebral artery occlusion.

(Highlights)
- Neumentix had an antioxidative effect.
- Neumentix kept the body weights in a stroke model.
- Neumentix reduced the infarction volume.

Abstract

Background: During an acute stroke, reactive oxygen species are overproduced and the endogenous antioxidative defense systems are disrupted. Therefore, antioxidative therapy can be a promising scheme to reduce the severity of stroke. Neumentix is a novel antioxidative supplement produced from a patented mint line and contains a high content of rosmarinic acid (RA). Although Neumentix has proven diverse efficacy and safety in clinical trials, its effect on strokes is unclear.

Methods: Mice that were treated with Neumentix or vehicle for 14 days underwent transient middle cerebral artery occlusion (tMCAO) for 60 min. Mice were sacrificed 5 days after tMCAO.

Results: Neumentix preserved body weight after tMCAO, showed a high antioxidative effect in serum, and reduced infarction volume compared to the vehicle. The expression of 4-hydroxy-2-nonenal, Nε-(carboxymethyl) lysine, and 8-hydroxy-2′-deoxyguanosine was reduced in Neumentix-treated mice.

Conclusion: The antioxidative effect of Neumentix was confirmed. This is the first report to demonstrate the antioxidative effect of Neumentix on strokes.

Introduction

Stroke is the leading cause of disability and death worldwide (Roth et al., 2018), but current available therapies are limited (Saver et al., 2015). Oxidative stress is the major component of the stroke cascade, especially in the acute phase of an ischemic stroke (Abe et al., 1988; Moretti et al., 2015). During brain ischemia and reperfusion, reactive oxygen species (ROS), also referred to as oxygen free radicals, are overproduced, while the endogenous antioxidative defense systems are disrupted (Chen et al., 2011). ROS generates toxic aldehyde 4-hydroxynonenal (4-HNE) (Mehta et al., 2007), glycated protein N(epsilon)-(carboxymethyl) lysine (CML) (Schleicher et al., 1997), and oxidized DNA 8-hydroxy-2-deoxyguanosine (8-OHdG). Thus, antioxidative therapy can be a promising means of reducing the severity of strokes.

We already reported an antioxidative drug, edaravone, as an excellent neuroprotective free radical scavenger (Abe et al., 1988; Yamashita et al., 2009). Antioxidative dietary supplements also suggest their efficacy against strokes in animal stroke models (Kusaki et al., 2017; Shang et al., 2018). Neumentix is a strong antioxidative dietary supplement produced from a patented mint line. The mint line was developed through traditional breeding techniques to contain higher amounts of naturally-occurring rosmarinic acid than other commercially available spearmint extracts (Falcone et al., 2018).

In the present study, therefore, we evaluated a possible effect of Neumentix against strokes, focusing on the antioxidative aspect.

Experimental Procedures

Animal sample preparation (serum and brain sections)

All mice were treated based on a procedure approved by the Animal Committee of the Okayama University Graduate School of Medicine (OKU-2018910). All experiments were carried out in accordance with ARRIVE guidelines and the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23).

Adult male C57BL/6J mice (23-27 g, 7 week old) were purchased from SLC, Japan (Shizuoka, Japan). The mice were housed in a temperature-regulated room (23-25°C) with free access to food and water, under a 12 hr light/12 hr dark cycle. The mice were randomly assigned to either the vehicle group (physiological saline, i.p., n = 35) or the Neumentix (134 mg/kg/d, containing RA 20 mg/kg/d, i.p., n = 31) group. Each group included three sham operation mice.

At the age of 8 weeks, mice in the vehicle group or mice in the Neumentix group were administered to a 14 day pre-treatment. Each group included sham operation mice. Neumentix is a phenolic compound that contains approximately 15% rosmarinic acid in addition to a number of other classes of phenolic compounds including salvianolic, caffeoylquinic, and hydroxyphenyl propanoic acids (Nieman et al., 2015). The other component of Neumentix is protein, carbohydrate, and dietary fibers, etc. Neumentix was dissolved in saline (1% of each mouse's weight) and intraperitoneally injected into each mouse.

On the 15th day of the pre-treatment, the mice were subjected to transient middle cerebral artery occlusion (tMCAO) (Yamashita et al., 2006). During surgery, the mice were anesthetized with a mixture of nitrous oxide/oxygen/isoflurane (69/30/1%) with an inhalation mask. Mouse body temperature was maintained at 37 ± 0.3°C on a heating pad (BWT-100; Bio Research Center, Aichi, Japan). Once the right common carotid artery (MCA) was exposed, a nylon thread with a silicon-coated tip was inserted into the right middle cerebral artery. After 60 min of occlusion, the silicon-coated thread was pulled out to reperfuse the blood flow of MCA. Sham operations were performed in the same way except for the nylon thread insertion. After tMCAO or sham operation, animals received the after-treatment of vehicle or Neumentix once a day for a period of 5 days (Fig. 1).

Five days after tMCAO, mice were deeply anesthetized by intraperitoneal injection of pentobarbital (40 mg/kg), and blood was collected from their hearts. Serum samples were separated from whole blood by centrifugation (10 min, 4°C) and stored at -80°C for d-ROMs and the OXY-Adsorbent test. The anesthetized mice's brains were perfused with chilled phosphate-buffered saline (PBS, pH 7.4) and 4% paraformaldehyde solution. The brains were removed and immersed in the 4%

paraformaldehyde solution overnight at 4°C. After fixation, brains were incubated in 10% sucrose solution for 24 hrs and then incubated in 30% sucrose solution for 48 hrs at 4°C. The fixed brains were frozen in liquid nitrogen and stored at −80°C. Coronal 20 μm-thick brain sections were prepared using a cryostat (HM525 NX, Thermo Fisher Scientific, Waltham, MA, USA) at −20°C for use in staining. This experiment is a part of a larger project focusing primarily on the effects of Neumentix (Bian et al., 2020, in submission).

Body weight and a rotarod test

The body weight of mice was measured 14, 8, and 1 days before tMCAO, and 1, 3, and 5 days after tMCAO. A rotarod test was also conducted based on a previous test method in our laboratory (Ohta et al., 2006; Kawai et al., 2010). Mice were placed on the treadmill (MK 132 670; Muromachi Kikai Co., Tokyo, Japan), and the speed was increasing from 1 rpm to 45 rpm. The mice ran on the treadmill until they fell off or up to 300 sec. Running time was assessed for motor functions. Rotarod test results were recorded on 14 and 1 days before tMCAO, on the day of tMCAO, and 1, 3, and 5 days after tMCAO.

Antioxidative markers in serum

The antioxidant capacity of mice serum was assayed by an OXY-Adsorbent test kit (Diacron International, Grosseto, Italy). The serum from each mouse was mixed with a strong ROS, hypochlorous acid (HOCl), which induces an antioxidative reaction against HOCl, and is the subsequent decrease of HOCl in the serum. A coloring agent was then added to the serum-HOCl mixture. The remaining HOCl which was not erased by the antioxidative effect of the serum, reacted to the coloring agent. The spectrophotometer (Free Radical Elective Evaluator, Diacron International, Grosseto, Italy) automatically calculated antioxidant capacity as the level of erased HOCl by the serum (μmol HOCl/mL). (Tamaki et al., 2011).

Oxidative stress was examined by the d-ROMs test kit (Diacron International) in accordance with the manufacturer's instructions. In brief, the serum from each mouse was mixed with acetate buffer and coloring agent (chromogen). The serum was then checked by a spectrophotometer, showing a ROS level as "Carratelli units" (CARR U). One CARR U corresponds to 0.08 mg per 100 mL of H_2O_2 (Tamaki et al., 2011).

Hematoxylin eosin staining and infarct volume

Coronal brain sections (20 μm) were stained as hematoxylin eosin staining. The infarction area was detected by microscopy (SZX-12; Olympus Optical, Tokyo, Japan) and computer software, Image J (NIH, Bethesda, MD, USA). The infarction volume in each mouse was calculated by summing the infarction volumes in three serial brain sections, at a 0.5 mm interval, between 0.5 mm anterior and 0.5 mm posterior to the bregma as previously reported (Nakano et al., 2017).

Oxidative stress marker expression in brains

Immunohistochemistry of 4-HNE, CML, and 8-OHdG was performed. Brain sections were dried and incubated in 0.3% H_2O_2/methanol for 20 min and 5% bovine albumin was blocked on the section for 1 hr. After washing in PBS, sections were incubated at 4°C overnight using the following

primary antibodies: mouse 4-HNE (1:50; JalCA, Shizuoka, Japan, MHN-100P, AB_1106813), mouse CML (1:200, Cosmo Bio, Tokyo, Japan, AGE-M01, AB_10705361) and mouse 8-OHdG (1:20; JalCA, MOG-100P, AB_1106818). Negative control sections were stained without primary antibodies.

After overnight incubation, sections were washed in PBS and incubated with biotinylated anti-mouse IgG secondary antibodies (Vector Laboratories, Burlingame, CA, USA) diluted at 1:500 for 2.5 hrs at room temperature. Immunoreactivity for each antibody was developed in a horseradish peroxidase streptavidin–biotin complex solution (VECTASTAIN ABC Kit, Vector Laboratories) and then incubated with 0.04% 3,3'-diaminobenzidine tetrahydrochloride (DAB).

Statistical analysis

In order to count the number of positive cells obtained from immunohistochemistry, three serially DAB-stained brain sections were selected from each mouse brain (from 0.5 mm anterior and 0.5 mm posterior to the bregma). From each section, three peri-ischemic areas were randomly captured at 200× magnification with a microscope (BX51; Olympus, Tokyo, Japan). Data were analyzed in GraphPad Prism (version 8.0, GraphPad Software Inc., San Diego, CA, USA). An unpaired student's *t*-test was used for all statistical analyses. Values were means ± standard deviation. Significant differences were described at $*p<0.05$ and $**p<0.01$.

Results

Body weight and a rotarod test

Neumentix-treated mice maintained their body weights after 5 days of tMCAO, compared to vehicle-treated mice (vehicle = -23.9 ± 5.9% vs Neumentix = -15.2 ± 11.6%, *p<0.05). The change in weight was described as the rate of change in weight from one day before tMCAO.

In the rotarod test, Neumentix-treated mice tended to keep their motor function more than vehicle-treated mice (vehicle = 177.0 ± 96.0 sec vs Neumentix = 222.0 ± 102.9 sec), but the difference was not significant (Fig. 2).

Antioxidative markers in serum

The OXY-adsorbent test revealed that serum from Neumentix-treated mice had a significantly stronger antioxidative effect (371.2 ± 54.6 μmol HClO/mL, **p<0.01 vs vehicle) than that from vehicle mice (299.7 ± 50.7 μmol HClO/mL) (Fig. 3a).

The d-ROMs test demonstrated that serum from Neumentix-treated mice showed significantly less oxidative stress (111.3 ± 25.7 CARRU, *p<0.05 vs vehicle) than serum from vehicle-treated mice (135.7 ± 18.8 CARRU) 5 days after tMCAO (Fig. 3b).

Hematoxylin eosin staining and infract volume

The brains of Neumentix-treated mice showed a smaller infarction volume (11.7 ± 5.5 mm^3, **p<0.01 vs vehicle) than the brains of vehicle-treated mice (18.7 ± 2.8 mm^3) (Fig. 4).

Oxidative stress marker expressions

Three antioxidative stress markers (4-HNE, CML, and 8-OHdG) were strongly expressed in the peri-infarct areas. 4-HNE was mainly labeled in the cytoplasm of cerebrocortical cells after 5 days of tMCAO. The cells of Neumentix-treated mice showed lighter staining than the vehicle-treated mice. CML was relatively clearly labeled in the cytoplasm of cerebrocortical cells after 5 days of tMCAO. 8-OHdG was mainly labeled in the cytoplasm and a few cells are stained in the nucleus 5 days after tMCAO. The brain slices obtained from sham operation mice presented only a few positive cells for the three antioxidative stress markers.

Quantitative analysis revealed that Neumentix-treated mice showed fewer positive cells for the three antioxidative stress markers, 4-HNE (vehicle = 130.1 ± 16.2 vs Neumentix = 82.9 ± 21.5/mm^2, **p<0.01), CML (vehicle = 123.8 ± 21.4 vs Neumentix = vs 90.4 ± 20.6/mm^2, **p<0.01) and 8-OHdG (vehicle = 113.5 ± 18.5 vs Neumentix = 85.7 ± 6.2/mm^2, **p<0.01). There was no significant difference between the brains of vehicle-treated sham mice and Neumentix-treated sham mice (Fig. 5).

Discussion

RA has been reported as very effective for treating memory deficits in ischemic mice (Fonteles et al., 2016; Cui et al., 2018) and is widely known for its anti-obesity, anti-apoptosis, and anti-aging

effects, among others (Nadeem et al., 2019).

RA is the main component of Neumentix, which maintained the body weight of mice 5 days after tMCAO in the present study (Fig. 2). The OXY-adsorbent test and the d-ROM test revealed that Neumentix had an antioxidative effect and reduced ROS in blood serum (Fig. 3). Neumentix treatment reduced brain infraction volume (Fig. 4) and the expression of three oxidative stress markers (Fig. 5).

The antioxidative effect of Neumentix is mainly derived from its main component, RA. The structure of RA has the direct antioxidative effect. The hydroxyl groups of RA give electrons to free radicals in the body making the free radicals non-toxic (Lee et al., 2016). RA has four hydroxyl groups that lie adjacent to each other, a structure that has a high antioxidative effect (Villaño et al., 2007). Therefore, RA was expected to have greater antioxidative power than other phenols. The indirect effect of RA was also reported. In a stroke model mice experiment, RA upregulated Nrf2 and HO-1. Nrf2 is a transcriptional factor with an antioxidative enzyme group and HO-1 is its downstream molecule. Inhibition of the PI3K/Akt pathway, which is the upstream pathway of Nrf2, downregulated Nrf2 expression (Cui et al., 2018). RA activated Nrf2 and HO-1 via the PI3K/Akt pathway. The activation of Nrf2 and HO-1 led not only to an increase of antioxidative enzyme, but also to a decrease of NF-κB, Cox2, etc. This decrease suppressed excessive inflammation and conserved neurons and the blood brain barrier (Minhaj et al., 2017; Oliveira et al., 2019). In addition, RA upregulated Bcl-2 expression and downregulated Bax in stroke model mice experiments while reducing the dopamine degradative enzyme and increasing dopamine concentration in the brain (Hase et al., 2019). Thus, RA is expected to work as a preventive agent for vascular parkinsonism after strokes.

RA can pass the blood brain barrier and then effectively scavenge toxic oxidative radicals if it is administered intraperitoneally (Falé et al., 2011) especially when the blood brain barrier is disrupted due to stroke (Kondo et al., 1997). Neumentix improved agility in young human subjects and attentional ability in old subjects (Falcone et al., 2018). In addition to age-associated memory impairment, the quality of working memory and spatial working memory were improved in Neumentix clinical trials (Nieman et al., 2015; Herrlinger et al., 2018).

In addition, Neumentix consists of not only RA, but also more than 50 other unique phenols, as phytochemicals. The combination of various natural phytochemicals in plants, which protect plants naturally against environmental stress, often shows synergistic effects (Karimi et al., 2015). Therefore, Neumentix is expected to be more effective than a mono-therapy of RA against oxidative stress. Daily intake of antioxidative supplements can be a good preventive measure against strokes. Neumentix is manufactured from mint, which is widely used as food, and has historically proven safety. The safety of Neumentix was also confirmed by several genotoxicity and animal studies. Currently, four human clinical trials of Neumentix have been conducted. For instance, the results of a 90-day double-blined clinical trial of supplementation with Neumentix revealed no serious adverse events in human subjects (Falcone et al., 2018). Therefore, Neumentix is suitable for long-term pre-treatment.

In our mice experiment, we used intraperitoneal administration because the amount of intake by mice might be unstable if orally ingested. Regarding human use, Neumentix also showed an effect by daily oral intake. For example, in a clinical trial, oral intake of Neumentix significantly improved the quality of working memory and spatial working memory (Nieman et al., 2015; Herrlinger et al., 2018). For clinical use, oral intake would be an appropriate route of daily administration.

In conclusion, this is the first report to demonstrate Neumentix as an effective, novel, and

safe ROS scavenger for the reduction of stroke onset risk and symptom severity.

Acknowledgments

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Conflict of Interest

The authors disclose no potential conflicts of interest.

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Figure legends

Figure 1. Experimental protocol

Mice were divided into the vehicle or Neumentix group. Black arrows indicate intraperitoneal injection of saline or Neumentix. Mice received 14 days of pre-treatment, followed by 60 min tMCAO. After 5 days of tMCAO, mice were sacrificed and brain samples were obtained.

Figure 2. Body weight and rotarod test

Body weight was described as the rate of change (%) compared to the weight one day before tMCAO. Compared with vehicle mice, Neumentix-treated mice maintained their weight. Values are means ± S.D. (*p<0.05, **p<0.01; student's t-test).

Figure 3. Antioxidative markers in serum

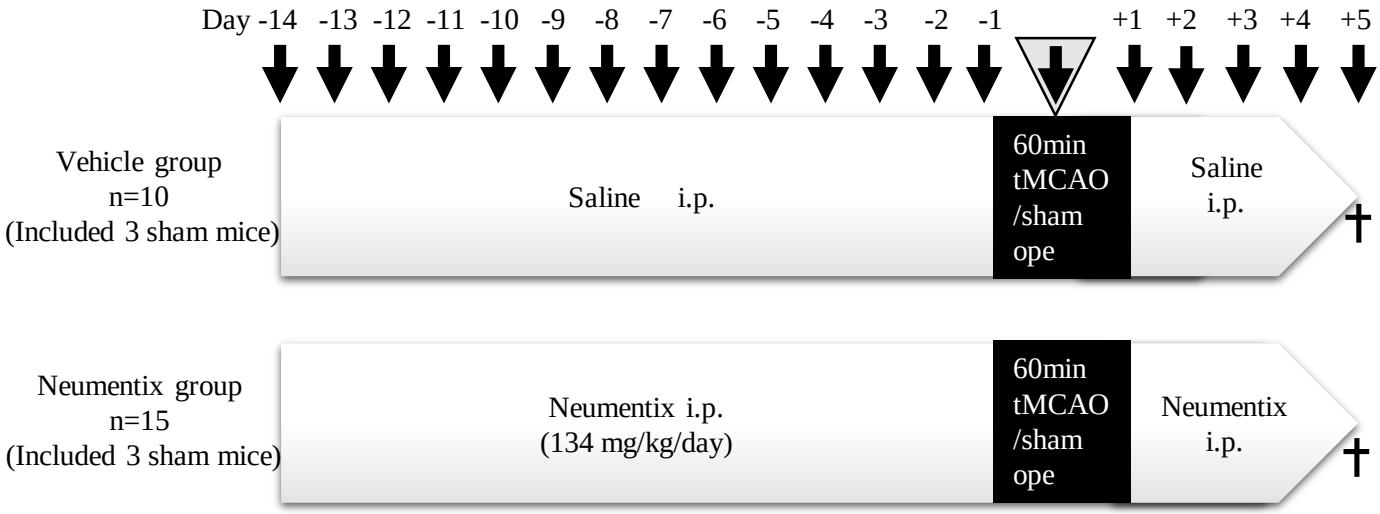
Serum levels of antioxidant capacity (OXY-adsorbent test) and oxidative stress (d-ROMs test). Note the significantly high antioxidant capacity and oxidative stress reduction in Neumentix-treated mice (*p<0.05, **p<0.01).

Figure 4. Hematoxylin eosin staining and infarct volume

HE staining at 5 days after tMCAO, and quantitative analysis of cerebral infarct volume. Infarct areas are marked as a dotted line. Scale bars: 1 mm.

Figure 5. Antioxidative marker expressions in brains

Representative photomicrographs of 4-HNE, CML, and 8-OHdG expression (left) and the number of positive cells (right). Note the significant reduction in positive cells for the three markers in Neumentix-treated mice (**p<0.01). Scale bars: 100 μm.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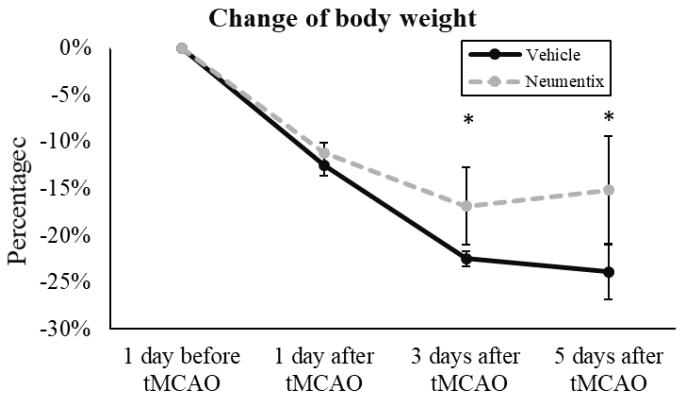
Vehicle group
n=10
(Included 3 sham mice)
Saline i.p.
60min tMCAO /sham ope
Saline i.p.
†
Neumentix group
n=15
(Included 3 sham mice)
Neumentix i.p.
(134 mg/kg/day)
60min tMCAO /sham ope
Neumentix i.p.
†

Fig.1


Change of body weight
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Neumentix
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5 days after tMCAO

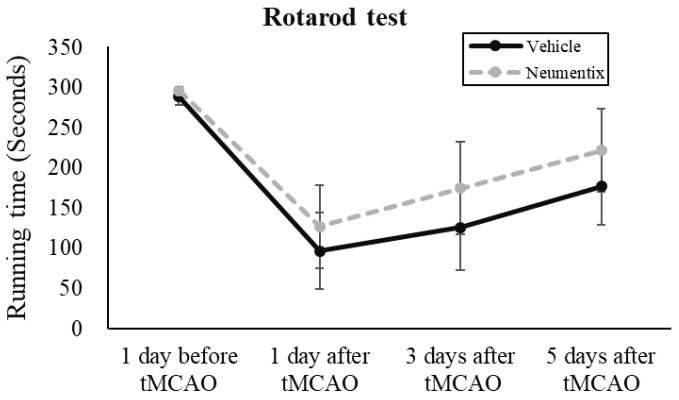

Rotarod test
350
300
250
200
150
100
50
0
Running time (Seconds)
Vehicle
Neumentix
1 day before tMCAO
1 day after tMCAO
3 days after tMCAO
5 days after tMCAO

Fig.2

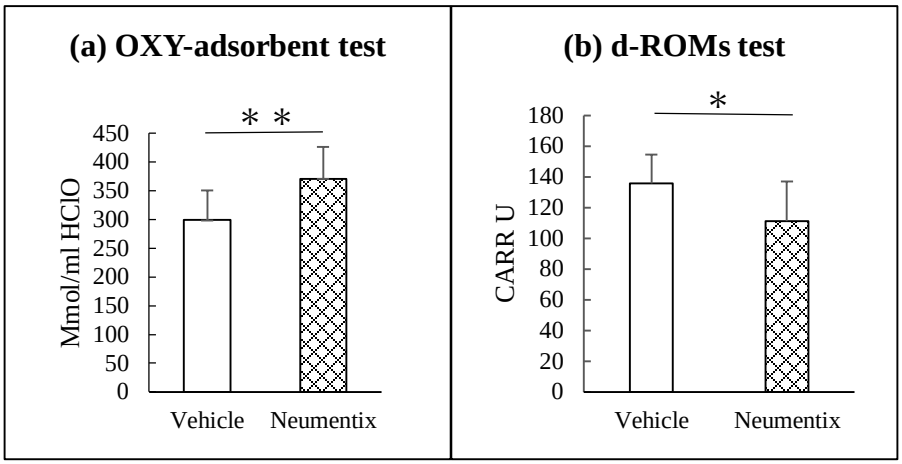
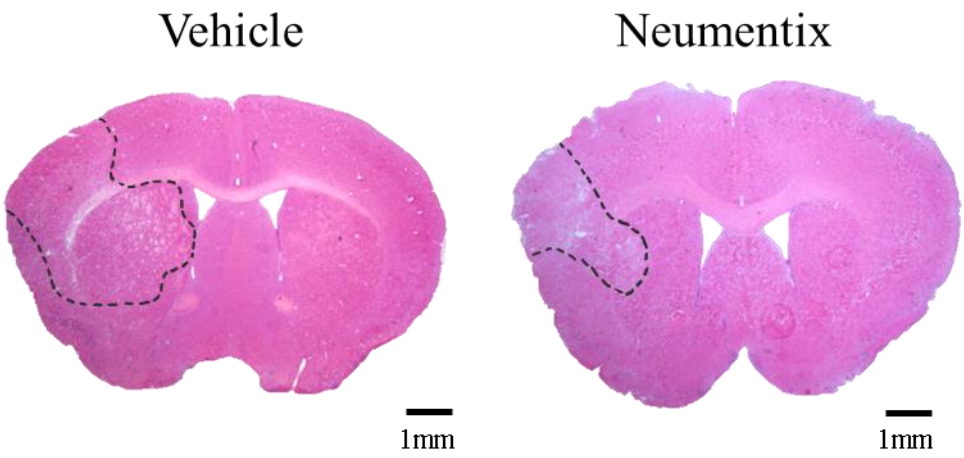

(a) OXY-adsorbent test
Mmol/ml HClO
450
400
350
300
250
200
150
100
50
0
＊＊
Vehicle
Neumentix
(b) d-ROMs test
CARR U
180
160
140
120
100
80
60
40
20
0
＊
Vehicle
Neumentix

Fig.3


Vehicle
Neumentix
1mm
1mm

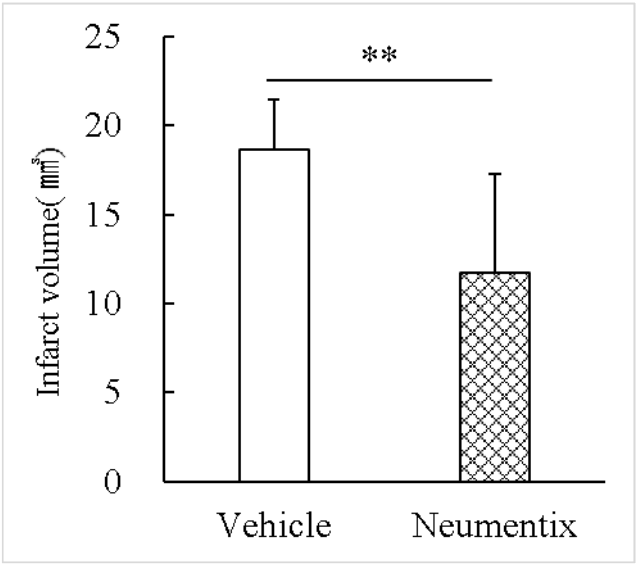

25
20
15
10
5
0
Infarct volume(mm³)
**
Vehicle
Neumentix

Fig.4

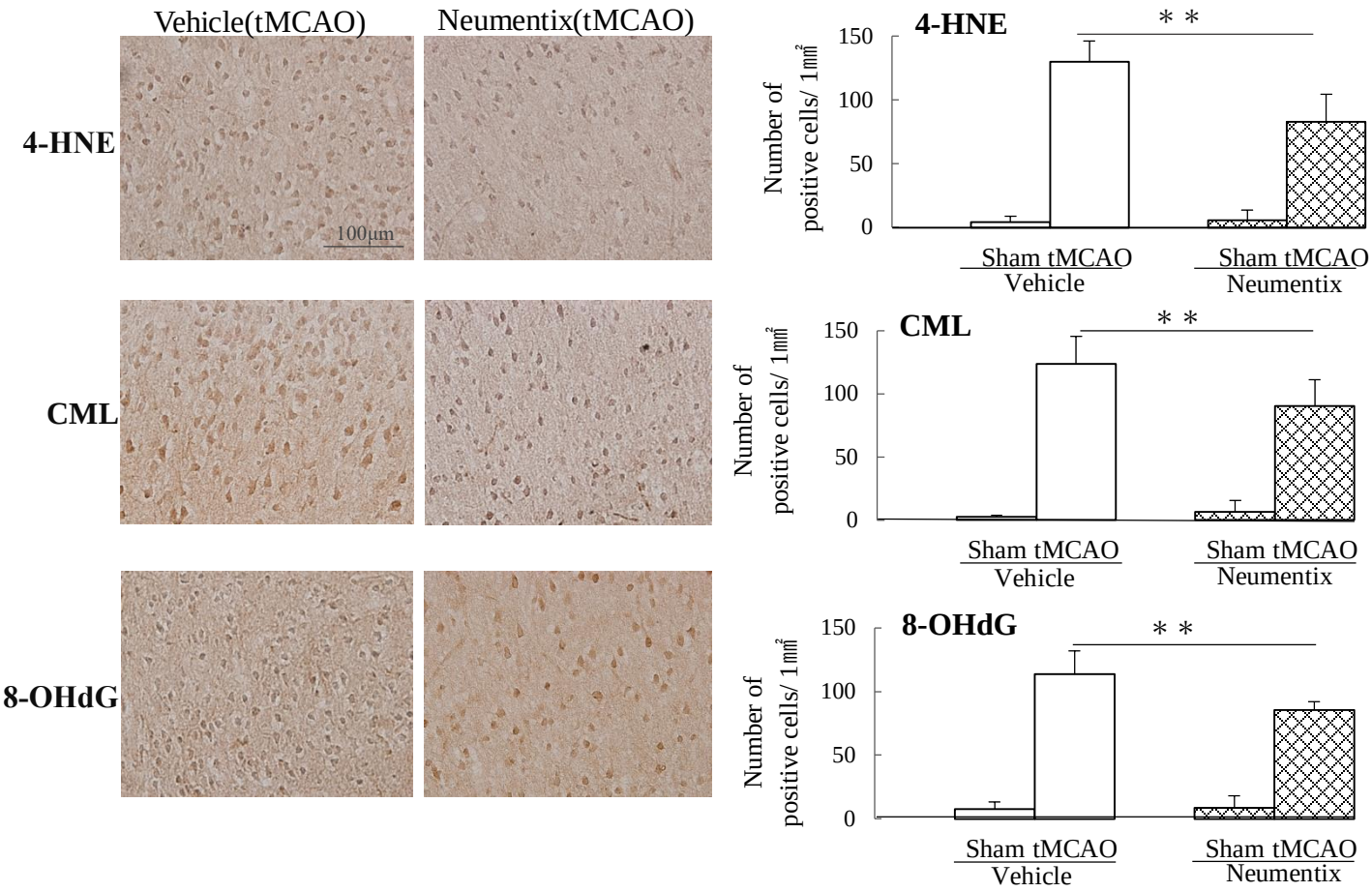

Vehicle(tMCAO)
Neumentix(tMCAO)
4-HNE
CML
8-OHdG
100μm
4-HNE
CML
8-OHdG
Number of positive cells/ 1㎟
150
100
50
0
＊＊
Sham tMCAO
Vehicle
Sham tMCAO
Neumentix

Fig.5