In TMT-B, we have to consider the effect of improvement of motor function on TMT-B execution time. However we found some improvements in scores about trunk and legs in UPDRS exam, not in arms and hands. Therefore the improvement in TMT-B must be derived from the improved executive function, not from motor function.

When evaluating cognitive task performances, the effects of learning on the task performances must be also considered; nevertheless, the impact of the learning effects on the improvements observed in this study was thought to be small because the second assessment was performed three months later.

The SDS scores improved in four of the five patients, although the differences between the baseline and follow-up scores were not significant; these findings suggest that rTMS may be effective for alleviating depression and mood disorders in Parkinson's disease. The application of rTMS over the left prefrontal area has been reported to improve intractable depression; although no significant changes in verbal, memory or intellectual function tests were reported, the TMT-B execution time decreased [31]. In healthy subjects, rTMS over the right prefrontal area also improved set switching, while rTMS over the left prefrontal area improved the Stroop test reaction time [32, 33]. Thus, as one aspect of executive function is improved and depression is alleviated, a relation between rTMS and neurological functions performed in the prefrontal area is indicated.

In recent years, studies have documented the long-term effects of low-frequency rTMS in several continuous sessions on different days, rather than single-session rTMS [34, 35, 36]. Shimamoto *et al.* [8] used a large circular coil to perform low-frequency rTMS over a broad area including the left and right motor, premotor and supplementary motor areas for a period of two months, and observed some improvements in the UPDRS. Mally *et al.* [37] also reported the long-term effects of rTMS. In the present study, improvements were observed after performing 100 stimulations per week for 3 months. In this manner, periodic stimulation over several months appeared to facilitate the reconstruction of the central nervous system, thus favorably impacting the cognitive function in Parkinson's disease.

While improvements were observed in ADL and the motor scores of the UPDRS, no improvements in the cognition-related scores were seen. The UPDRS has many motor function-related items, but few items for psychological and cognitive function involving impaired memory, orientation and mood. Hence, the UPDRS is suitable for assessing rTMS targeting the motor area, but not as suitable for assessing executive dysfunction, such as impaired set switching.

In four subjects, the 20 m walk time significantly decreased. The decrease in the 20-m walk time was particularly marked in Subject 3. In this subject, assessment using UPDRS revealed alleviation of rigidity of both lower extremities, suggesting that rTMS was effective against rigidity, as reflected by the marked decrease of the 20-m walk time.

In Parkinson's disease, the cortical silent period (CSP) is reportedly shortened [9, 38], indicating a disturbed inhibitory mechanism in the motor cortex. On the other hand, low-frequency low-intensity rTMS suppresses the motor cortex, and high-frequency high-intensity rTMS excites the motor cortex [39, 40]. It has also been documented that not only the site of stimulation, but also related areas away from the site of stimulation are excited [41]. Gerschlager *et al.* [42] performed subthreshold 1-Hz rTMS for a total of 1500 times each in the prefrontal cortex, premotor cortex, motor cortex and parietal cortex to suppress the site of stimulation, depressing the MEP amplitude. Significant suppression was observed with premotor stimulation, and suppression, albeit not significant, was seen with prefrontal stimulation. In the present study, the MEP amplitude during rTMS was less than 50 μV. In other words, the intensity might be subthreshold for the motor cortex. Thus, this suprathreshold low-frequency stimulation over the prefrontal area served as a subthreshold low-frequency stimulation over the supplementary motor and motor cortexes. While no significant differences were found, the results indicate that gait function also improves after rTMS. Because the motor cortex might be suppressed, so that gait function can be improved by rTMS.

In UPDRS, we found some improvements in motor exam, especially in in tremor at rest in legs, rigidity in legs, posture and body bradykinesia as mentioned above. These results might be due to the same reason we mentioned in 20 m walk time. rTMS might be subthreshold low-frequency stimulation over the supplementary motor and motor cortexes as mentioned above. Therefore rTMS improved motor function of trunk and legs in Parkinson disease, so that the gait function might improve.

The improvements in the executive function test suggest improvements in the subjective symptoms and objective findings. These results indicate that delays in the start of movements within ADL in Parkinson's disease, such as a lack of smoothness in conversations, a slowness of movements, and frozen gait, are closely correlated with executive dysfunction.

In the present study, low-frequency suprathreshold stimulation over the prefrontal area was effective for executive function and ADL, allowing functional failure in the frontostriatal circuit to recover [43, 44]. Delong, Alexander and others [45, 46, 47 and 48] have described five circuits in mammals (motor circuit, ocular movement circuit, dorsolateral prefrontal circuit, lateral fronto-orbital circuit and anterior cingulate gyrus circuit); they reported that a closed circuit was formed through communications with certain areas of the cerebral cortex and basal ganglia. Three circuits, in addition to the motor and ocular movement circuits, were then combined as the cognition loop (prefrontal circuit and limbic circuit). Hence, rTMS over the bilateral dorsolateral prefrontal area may trans-synaptically affect the frontostriatal circuit, particularly the prefrontal circuit.

In this study, the bilateral dorsolateral prefrontal areas in Parkinson's disease were simultaneously stimulated with a low-frequency suprathreshold stimulation. Cognitive tests suitable for evaluating the prefrontal area were used to assess the effects of stimulation. In addition, the use of a circular concave coil enabled

relatively localized sites to be stimulated, with EEG monitoring for the safety.

In the future, the long-term therapeutic effects of rTMS, particularly with regard to the frequency, stimulation intensity and rTMS coil-type, need to be investigated. Low-frequency suprathreshold rTMS in Parkinson's disease improved cognitive function and symptoms related to the prefrontal area. Hence, when combined with drug therapy and rehabilitation, rTMS appears to be useful for maintaining and improving function. Further developments related to the application of rTMS in Parkinson's disease are expected.

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Research Report

Simplified experimental cerebral aneurysm model in rats: Comprehensive evaluation of induced aneurysms and arterial changes in the circle of Willis

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ABSTRACT

Establishing a simple and comprehensive experimental model is one of the most important issues in the study of cerebral aneurysms. Previous models in the rat required two stage surgeries and observations were limited to a few branching sites. The present study aimed to introduce a simplified model in rats and to provide a comprehensive evaluation of induced arterial changes in the circle of Willis. Sprague–Dawley rats underwent ligation of the right common carotid artery, and posterior and inferior (group 2, n=9) or only posterior (group 3, n=12) branches of the bilateral renal arteries, and bilateral oophorectomy. Dahl salt-sensitive rats underwent only carotid ligation and bilateral oophorectomy (group 5, n=11). All surgical procedures were completed in one procedure instead of two in the original method. Salt loading was started after the surgery. Five rats of each strain without treatment served as controls (groups 1 and 4, respectively). Three months later, vascular corrosion casts of the cerebral arteries were examined by scanning electron microscopy. Experimental rats in groups 2, 3, and 5 developed 43 aneurysmal lesions at branching sites. Forty-eight arterial changes including dilatation, tortuosity, and fusiform or lateral wall aneurysms were observed at non-branching sites. Group 3 appeared to be superior to the other groups for experimental studies. The frequency and degree of the induced lesions were comparable with previous studies even after the surgical simplification. The present model may be more practical for the study of experimental cerebral aneurysms.

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Abbreviations: A1, proximal segment of the anterior cerebral artery before forming the azygos artery; ACA, anterior cerebral artery; AcoA, anterior communicating artery; BAPN, beta-aminopropionitrile; BUN, blood urea nitrogen; CCA, common carotid artery; ICA, internal carotid artery; OA, olfactory artery; P1, proximal segment of the posterior cerebral artery between the origin from the basilar terminal and junction with the posterior communicating artery; PCA, posterior cerebral artery; PcoA, posterior communicating artery; SABP, systolic arterial blood pressure; SAH, subarachnoid hemorrhage; S/D, Sprague–Dawley

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1. Introduction

Aneurysmal subarachnoid hemorrhage (SAH) continues to present challenging problems to neurosurgeons in spite of the advances in neurosurgery. SAH occurs in 10.5–23 patients per 100,000 persons per year (Inagawa et al., 1995; Linn et al., 1996). Rupture of an intracranial aneurysm accounts for 85% of cases (van Gijn and Rinkel, 2001). Only 10% of cases are identified prior to rupture and 11–14% of all patients with SAH will die before receiving medical attention (Huang and van Gelder, 2002). The mortality rate is still significant even after hospitalization and treatment, so the population-based fatality rate of SAH may be as high as 32–67% (Hop et al., 1997). Consequently, much effort has been expended to clarify the mechanisms underlying the formation, growth, and rupture of aneurysms, and to find a way to prevent this sequence of events. Various animal models for induction of cerebral aneurysms have been developed using rats (Coutard and Osborne-Pellegrin, 1997; Handa et al., 1983; Hashimoto et al., 1979a; Hashimoto et al., 1979b; Hashimoto et al., 1980; Hashimoto et al., 1984; Jamous et al., 2005a; Jamous et al., 2005b), mice (Coutard, 1999; Morimoto et al., 2002), and monkeys (Hashimoto et al., 1987).

Arterial hypertension is generally thought to be very important in the formation and rupture of saccular aneurysms in humans (de la Monte et al., 1985; Stehbens, 1989) and animals (Hashimoto et al., 1987; Nagata et al., 1980). Induced hypertension by renal artery ligation combined with carotid ligation has been used to enhance hemodynamic stress on the collateral pathway along the circle of Willis, finally resulting in aneurysmal changes (Handa et al., 1983; Jamous et al., 2005a; Jamous et al., 2005b, Nagata et al., 1980).

Recently, bilateral oophorectomy combined with induced hypertension and carotid ligation in the rat was introduced to decrease estrogen levels, which functions to maintain arterial integrity, and showed increased susceptibility to aneurysm formation (Jamous et al., 2005b; Jamous et al., 2005c). This experimental model is promising with a significant incidence of aneurysms compared to previous studies (Handa et al., 1983; Hashimoto et al., 1979a; Hashimoto et al., 1979b; Hashimoto et al., 1980; Hashimoto et al., 1984; Nagata et al., 1980). However, two stage surgery is necessary, first for ligation of the renal and carotid arteries, and second for oophorectomy. The model was mainly evaluated in the anterior circulation, especially the anterior cerebral artery (ACA)-olfactory artery (OA) branching site opposite the carotid ligation, although aneurysmal changes are known to develop in the posterior circulation (Handa et al., 1983; Hashimoto et al., 1980; Kondo et al., 1997; Nagata et al., 1980). Simplification of the surgical procedure and comprehensive analysis of the effects on the circle of Willis in rats may promote further research of experimental cerebral aneurysms.

We describe our method of one stage surgery which achieves both ligation of the bilateral renal arteries and right common carotid artery (CCA), and bilateral oophorectomy. Our study also investigated all sites of the circle of Willis which suffer excess hemodynamic stress to comprehensively classify the various aneurysmal changes using vascular corrosion casts examined under a scanning electron microscope. The degree of hypertension (Nagata et al., 1980) and rat strains (Coutard and Osborne-Pellegrin, 1997) are the major factors affecting frequency of aneurysm induction, so we also compared different methods of renal artery ligation in Sprague–Dawley (S/D) and Dahl salt-sensitive (Dahl et al., 1962) rats.

2. Results

2.1. *Lesions at branching sites*

The experimental groups were explained in Table 1.

Branching sites with increased hemodynamic stress in the collateral circulation after right CCA ligation were investigated, along the left internal carotid artery (ICA)-A1 pathway and along the basilar artery-right P1-posterior communicating artery (PcoA)-ICA pathway. None of the S/D (group 1) or Dahl salt-sensitive (group 4) control rats showed any lesions at these branching sites.

Branching site lesions were classified in 4 stages as shown in Fig. 1 according to the previous papers (Jamous et al., 2005b; Jamous et al., 2005c). Table 2 shows the 43 lesions detected at branching sites. Groups 2 and 3 demonstrated 1.6 and 1.7 lesions/rat, respectively, and group 5 showed 0.8 lesions/rat ($P<0.05$). The left ACA-OA bifurcation was the most common site. Other less frequent sites were the left ICA bifurcation and left A1 fenestration. A few lesions developed at the origins of the small branches from the right P1.

Table 2 also shows the staging (Fig. 1) of these lesions. Distribution of staging showed significant differences between Groups 2, 3, and 5 ($P=0.036$, Chi-square test). Combining stages 2 and 3 showed a significant difference between Groups 3 and 5 ($P=0.02$), but not between Groups 2 and 3. Group 3 had more stage 3 aneurysmal changes than Group 5. All stage 3

Table 1 – Experimental groups.

Group	Strain	Number of rats	Renal artery (occluded branches)	Carotid artery	Oophorectomy	1% Saline for drinking
Group 1	S/D	5	–	–	–	–
Group 2	S/D	9	blt. posterior and inferior branches	rt CCA occlusion	blt. oophorectomy	+
Group 3	S/D	12	blt. posterior branches	rt CCA occlusion	blt. oophorectomy	+
Group 4	Dahl	5	–	–	–	–
Group 5	Dahl	11	–	rt CCA occlusion	blt. oophorectomy	+

blt.: bilateral, CCA: common carotid artery, rt: right, S/D: Sprague–Dawley.

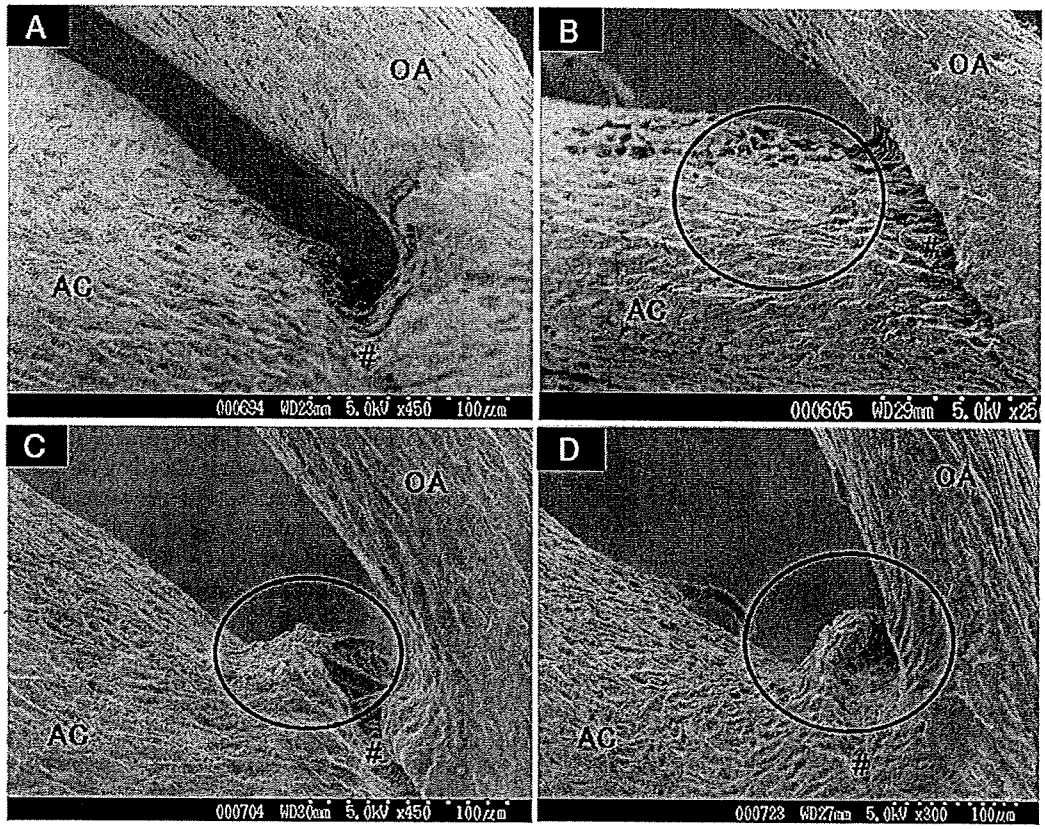

Fig. 1 – Classification of aneurysmal changes at the branching sites using scanning electron microscopy findings. (A) Normal endothelial cell imprints at the arterial bifurcation with no gross arterial dilatation compared to the contralateral side (stage 0 or normal). (B) Roughened apical intimal pad with irregularly shaped imprints (stage I). (C) Shallow fusiform elevation at the apical intimal pad covered with abnormal imprints (stage II). (D) Saccular aneurysm covered with abnormal imprints (stage III). ACA: anterior cerebral artery, OA: olfactory artery, #: nonpathological cleft always found in corrosion casts between ACA and OA. Distance between the adjacent dots at the lower right corner of A through D is 10 μm.

lesions were located in the anterior circulation, 5 at the left ACA-OA and 4 at the left A1 fenestration.

2.2. *Lesions at non-branching sites*

Non-branching sites with increased hemodynamic stress in the collateral circulation after right CCA ligation were also investigated for possible abnormal lesions. These lesions were classified according to the previous paper (Kondo et al., 1997) with minor modifications (Figs. 2 and 3). These lesions were eventually confined to the A1-communicating branches and right P1 segment (Table 3). None of the control rats (groups 1 or 4) showed any evidence of abnormality in these non-branching sites. None of the left A1 segments or right PcoA showed obvious abnormalities.

Groups 2, 3, and 5 demonstrated 1.7, 1.5, and 1.4 non-branching site lesions per rat, respectively, with no significant difference between the 3 groups (Table 3). Lesions of the A1-communicating branches developed in 58–78% of rats and lesions of the right P1 segment in 73–92% of rats. The sites and types of lesions showed no differences between Groups 2, 3, and 5.

2.3. *Development of hypertension and laboratory results*

Hypertension developed in all rats except the control groups (Groups 1 and 4) (Fig. 4A). The mean systolic arterial blood pressure (SABP) in the control animals was around 130 mm Hg. Groups 2 and 3 showed significant increases in SABP (186±6 and 187±8 mm Hg, respectively) at the end of the experiment compared with Group 1 (130±2 mm Hg). Dahl experimental rats (Group 5), which were given 1% saline without renal artery ligation, demonstrated only a modest but statistically significant increase in SABP (157±4 mm Hg) compared with Group 4 (127±2 mm Hg).

Serum total cholesterol levels increased in experimental rats compared with corresponding control groups (Fig. 4B), showing statistical significance in Groups 2 and 3. Serum blood urea nitrogen (BUN) (Fig. 4C) and serum creatinine (Fig. 4D) levels increased in all experimental groups, although significant increases were detected only between Groups 1 and 2. Serum renin (Fig. 4E) and angiotensin II (Fig. 4F) levels decreased in all experimental groups significantly except for rennin of Group 3 compared with the corresponding control groups. Estradiol (Fig. 4G) level showed tendency of decrease

Table 2 – Branching site lesions.

	Group 2	Group 3	Group 5
Number of rats	9	12	11
Total number of lesions	14	20	9
Number of lesions per rat (mean±SEM)	1.6±0.2	1.7±0.1	0.8±0.2
Site[a]			
No lesion	0 (0%)	0 (0%)	4 (36%)
Left ACA-OA	8 (89%)	12 (100%)	5 (45%)
Left A1 (fenestration or its branch)	1 (22%)	5 (42%)	1 (9%)
Left ICA bifurcation	4 (37%)	3 (25%)	0
Right PCA	1 (11%)	0 (0%)	3 (27%)
Staging[b]			
Stage 1	7 (50%)	5 (25%)	7 (78%)
Stage 2	6 (43%) #	7 (35%) #	2 (22%) #
Stage 3	1 (7%)	8 (40%)	0 (0%)

A1: proximal segment of the anterior cerebral artery, ACA: anterior cerebral artery, ICA: internal carotid artery, OA: olfactory artery, PCA: posterior cerebral artery, SEM: standard error of the mean.
[a] Percentages indicate incidence of lesions per rat.
[b] See text for staging description. Percentages indicate rate among total number of lesions. #: There were significant differences in the staging distributions between the 3 groups (P=0.036, Chi-square test). When stages 2 and 3 were combined, there was a statistically significant difference between Groups 3 and 5 (P=0.02), but not between Groups 2 and 3.

in all experimental rats, but did not reach statistically significant levels.

3. Discussion

The present simplified one stage procedure to perform both ligation of the CCA and renal arteries, and bilateral oophorectomy induced significant numbers and degree of experimental aneurysms comparable to those induced by the two-stage procedures in the experimental rat model (Jamous et al., 2005b; Jamous et al., 2005c). This study also comprehensively evaluated the various locations and types of induced aneurysmal/arterial changes around the circle of Willis.

3.1. *Experimental cerebral aneurysm models in rats*

The most frequently used experimental models of cerebral aneurysm in rats employ unilateral CCA ligation, beta-aminopropionitrile (BAPN, a lathyrogen) mixed in food, subcutaneous deoxycorticosterone injection, and salt loading in drinking water to induce hypertension (Handa et al., 1983; Hashimoto et al., 1979a; Hashimoto et al., 1979b). Macroscopic aneurysms were observed in 11 of 30 rats that died during the 11–21 weeks after the onset of BAPN intake (Hashimoto et al., 1979a). Subsequently, renal hypertension by ligating posterior branches of the bilateral renal arteries was combined with left CCA ligation, salt loading, and BAPN intake (Nagata et al., 1980). Eighteen macroscopic aneurysms were found in 13 rats at 16 weeks after the onset of the treatment (12 aneurysms on the P1 ipsilateral to the CCA ligation).

Recently, oophorectomy was used to induce aneurysm by reducing the estrogen level which is protective to arterial endothelial cells (Table 4) (Jamous et al., 2005b; Jamous et al., 2005c). Ligation of the right CCA and posterior branches of the bilateral renal arteries was performed in the first operation. One week later, salt loading in the drinking water was initiated. One month after the first operation, bilateral oophorectomy was performed in the second operation.

This model may be regarded as replacement of BAPN by estrogen deficiency to promote pathological changes in the arterial wall. The protective effects of estrogen for the arterial endothelium may be mediated at least partially by the enhancement of endothelial nitric oxide synthase expression and activity through antioxidant effects (Wagner et al., 2001). The vascular functions of nitric oxide include vasodilation (Furchgott and Zawadzki, 1980), inhibition of platelet aggregation (Alheid et al., 1987), and adhesion (Radomski et al., 1987), as well as inhibition of leukocyte adhesion (Kubes et al., 1991). Estrogen also inhibits nuclear factor κB (Galea et al., 2002), which plays a key role in the vascular inflammation as well as cerebral aneurysmal formation (Aoki et al., 2007).

Twelve microscopic aneurysms were induced in 9 (60%) of 15 rats at 3 months after the first surgery (Jamous et al., 2005b). Investigation of arterial branching sites of the anterior circulation contralateral to the CCA ligation found that the most frequent site for aneurysm development was the ACA-OA branching site (Jamous et al., 2005a; Jamous et al., 2005b; Jamous et al., 2005c).

One study systematically investigated both branching and non-branching sites (Table 4) (Kondo et al., 1997) with a model of renal hypertension and left CCA ligation followed by salt loading. At 12 months after the operation, 30 of 35 rats had developed 55 branching site aneurysms mainly at the right ACA-OA branching site, and 19 of 35 rats developed a total of 30 non-branching site aneurysms in the left P1 segment. The non-branching site lesions included fusiform or saccular aneurysms, and arterial changes could be divided into dilatation or tortuosity.

3.2. *Our experimental models and laboratory data*

In our experimental model, hypertension induced by renal artery ligation and salt loading, and hemodynamic stress caused by unilateral CCA ligation together with prolonged estrogen deprivation are considered to be main factors of induced aneurysmal changes.

Two types of renal artery ligation we compared in S/D rats; ligation of the posterior and inferior branches of the bilateral renal arteries in group 2, and ligation of only the posterior branches of the bilateral renal arteries in group 3. Laboratory data showed more renal dysfunction in group 2 compared with group 3 (Figs. 4C and D). Serum renin and angiotensin II levels were lower in both groups 2 and 3, more reduction in group 2 compared with group 3.

Renin secretion is currently believed to be controlled by the blood concentration of NaCl through the macula densa mechanism, sympathetic nerve function, pressoreceptors, and humoral factors such as the renin–angiotensin–

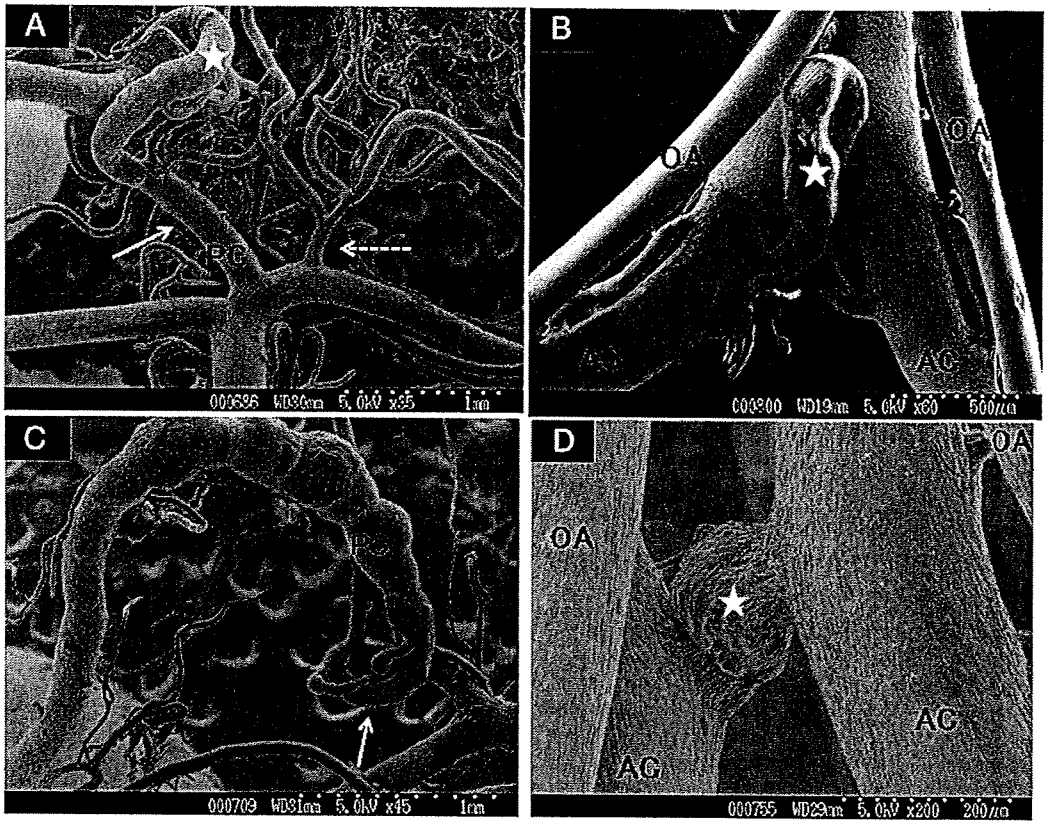

Fig. 2 – Classification of arterial changes at the non-branching sites using scanning electron microscopy findings. (A) Basilar tip complex showing dilatation (arrow) and tortuosity or looping (star) of the proximal segment (P1) of right posterior cerebral artery (PCA). Dotted arrow indicates the contralateral P1. (B) Dilatation and tortuosity (star) of the A1-communicating branch. (C) Dilatation and tortuosity or looping (arrow) of the right P1 with beaded like appearance. (D) Fusiform aneurysm (star) of the A1-communicating branch.

aldosterone system, vasopressin, prostaglandin, endothelin, nitric oxide, and others (Beierwaltes, 2006; Kurtz and Schweda, 2006). Salt loading induces hypertension by increase of circulating blood volume which in turn inhibits renin secretion due to suppression of pressoreceptors. Increased circulating blood volume due to higher salt sensitivity causes lower renin levels in the blood of Dahl salt-sensitive rats. Renin level is thought to increase in the acute stage of renal ischemia caused by renal artery occlusion, but renin secretion decreases after completion of renal infarction within the next few days

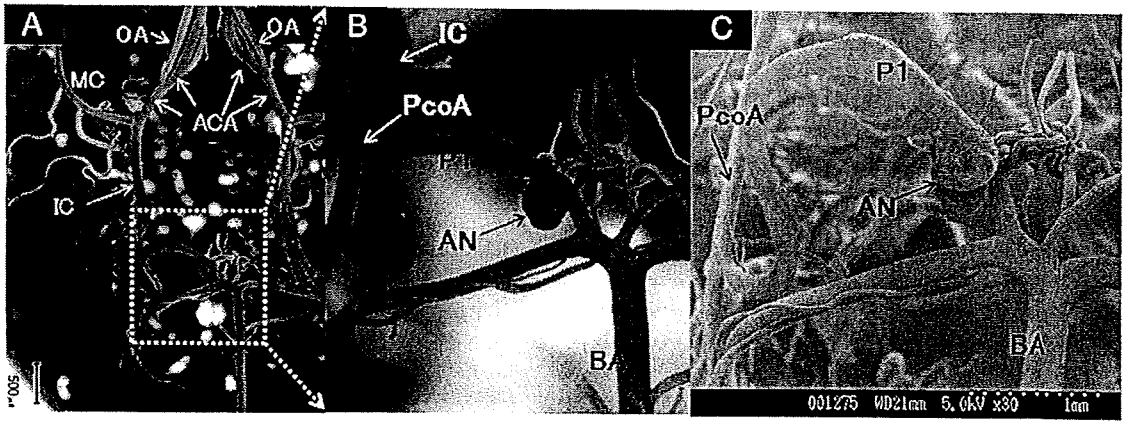

Fig. 3 – Light microscopy (A and B) and scanning electron microscopy (C) images of a cast of a representative lateral wall aneurysm. AN: aneurysm, BA: basilar artery, ICA: internal carotid artery, MCA: middle cerebral artery, PcoA: posterior communicating artery.

Table 3 – Non-branching site lesions.

	Group 2	Group 3	Group 5
Number of rats	9	12	11
Total number of arteries with abnormalities	15	18	15
Number of arteries with abnormalities per rat (mean ± SEM)	1.7 ± 0.2	1.5 ± 0.2	1.4 ± 0.2
Sites of arterial abnormality[a]			
No lesion	0 (0%)	0 (0%)	1
A1-communicating branch	7 (78%)	7 (58%)	7 (64%)
Right P1	8 (89%)	11 (92%)	8 (73%)
Type of abnormality[b]			
Dilatation	6 (40%)	10 (56%)	9 (64%)
Tortuosity	1 (7%)	2 (11%)	1 (7%)
Tortuosity + dilatation	3 (20%)	2 (11%)	3 (21%)
Fusiform aneurysm	4 (27%)	4 (22%)	2 (14%)
Lateral wall aneurysm	1 (7%)	2 (11%)	0 (0%)

A1: proximal segment of the anterior cerebral artery, P1: proximal segment of the posterior cerebral artery, SEM: standard error of the mean.

[a] Numbers of rats that demonstrated arterial abnormality at each site. Percentages indicate incidence of the lesions per rat. There was no significant difference in the site distributions between the 3 groups (Chi-square test).

[b] See text for type description. Numbers of each lesion type among total number of arteries with abnormality are shown. When a fusiform aneurysm was counted, dilatation was not counted for the same artery. Percentages indicate rate among total number of lesions. There was no significant difference in the type distributions between the 3 groups (Chi-square test).

(Lee and Shin, 1997). Gyton's pressure natriuresis theory predicts that larger renal infarction will cause lower blood renin levels (Hall et al., 1996). Our laboratory data detected lower renin and angiotensin II levels in group 2 than in group 3, compatible with these physiological expectation. The achieved levels of hypertension were not significantly different between the two groups, indicating that ligation of only the posterior branches as in group 3 may be both necessary and sufficient to induce renal hypertension.

In addition, mortality rate of group 2 (50%) was higher than that of group 3 (25%) probably due to more profound renal dysfunction. Together with the observation that incidence of total aneurysm was similar in groups 2 and 3 and that stage III aneurysm may be more frequent in group 3, it would be suggested that group 3 may be a candidate for further studies on experimental cerebral aneurysm in S/D rats.

Increased blood cholesterol levels in the experimental rats may be related to estrogen deficiency, but hypercholesterolemia is also reported in rats with chronic renal failure (Chmielewski et al., 2007). A twofold increase in sterol regulatory element-binding protein-2 messenger ribonucleic acid levels was found, which is regarded as the main regulator of cholesterol homeostasis. A twofold increase in liver cholesterologenesis rate was also noted in rats with chronic renal failure (Chmielewski et al., 2007). This may enhance the effect of hypertensive vasculopathy and induced endothelial dysfunction making the rat vasculature more prone to aneurysm formation.

In the present study, there were tendency of decrease in serum estrogen levels in groups 2, 3 and 5, but statistically significant difference was not reached compared with corresponding control groups. This may be accounted for by that the blood samples of groups 1 and 4 were obtained at a mean age of 9 weeks and those of groups 2, 3 and 5 were at 21 weeks, considering generally known age dependency of estrogen in females.

3.3. *Features and limitations of our experimental cerebral aneurysm model*

Most investigations of experimental cerebral aneurysms in rats have been limited to evaluating arterial bifurcations or branching sites. Our results show that 30 non-branching site aneurysms or arterial changes developed in 12 operated rats (group 2) in accordance with the previous report (Kondo et al., 1997). In addition, we demonstrated that arterial changes in the A1-communicating branches were rather frequent. These findings may imply that non-branching arterial changes in the P1 and the A1-communicating branches may also be a candidates for further investigations, especially of non-saccular aneurysms.

Branching site lesions were classified by the previous staging (Jamous et al., 2005c). However, discrimination between stages 0 and 1 was often difficult in contrast to stages 1 and 2, and stages 2 and 3 were straightforward in many instances. Further studies may combine stage 0 and 1 for simple expression of results if it does not compromise the purpose of the experiment.

This study showed that Dahl salt-sensitive rats can be used for an experimental cerebral aneurysm model, but the induced hypertension was moderate, and induced aneurysms were lower in both number and stage compared with Groups 2 and 3. Additive procedures to further elevate blood pressure such as ligation of the renal artery or increased salt loading may be recommended for the study of cerebral aneurysms.

In conclusion, experimental cerebral aneurysms were introduced using a simplified one stage procedure for ligation of the posterior branches of the bilateral renal arteries and unilateral CCA, together with bilateral oophorectomy. One stage surgery will be advantageous not only in saving time but also in an ethical point of view. Comprehensive evaluation of both branching and non-branching site lesions systematized the resulting aneurysmal changes. These induction techniques and evaluations are expected to become the basis for further research.

4. Experimental procedures

4.1. *Groups and surgical procedures*

The present experiments and protocols were conducted in accordance with the Japanese standards for the care and use of laboratory animals and were approved by the Animal Care Committee, Tohoku University Graduate School of Medicine. All surgical procedures were performed after induction of anesthesia by intraperitoneal injection of pentobarbital 40 mg/kg.

A total of forty-two 7-week-old female rats, consisted of 26 S/D and 16 Dahl salt-sensitive rats, were divided into 5

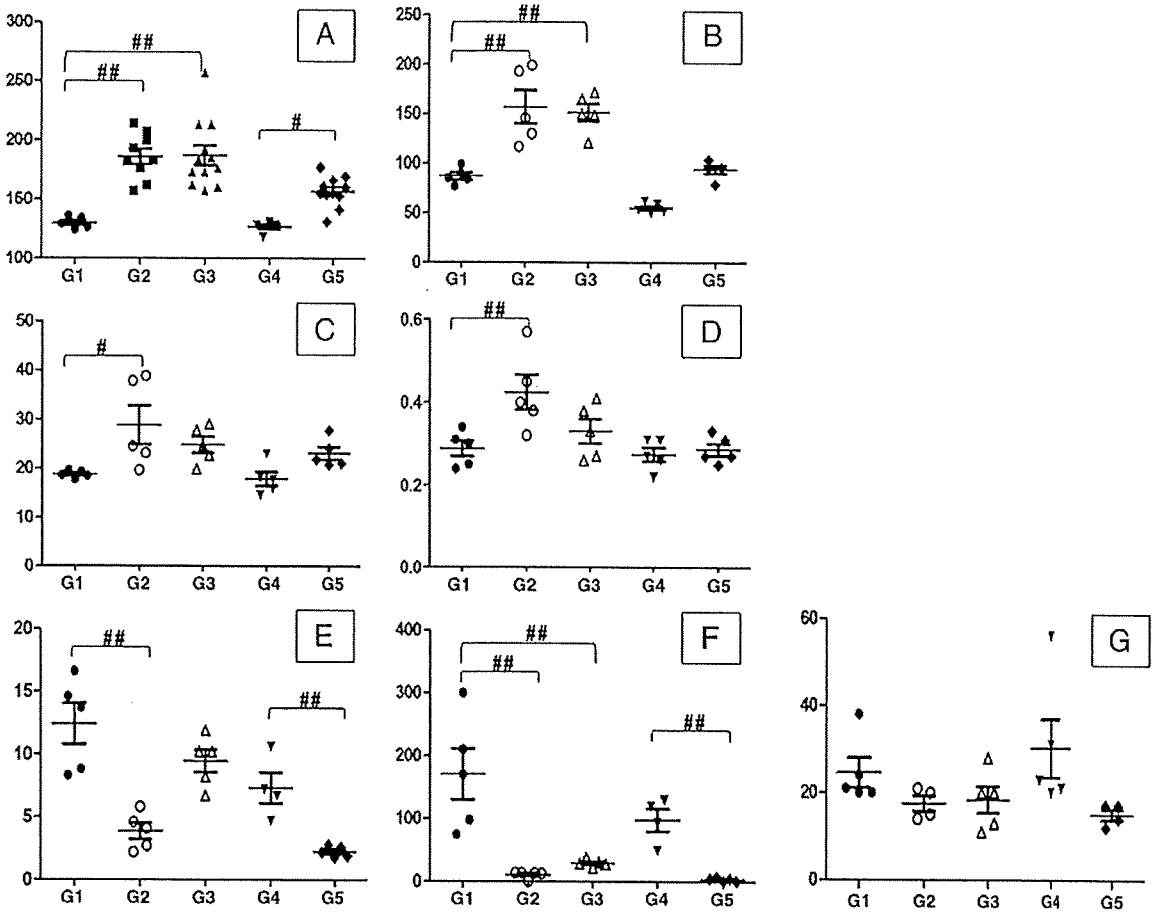

Fig. 4 – Summary of blood pressure (A) and blood sample (B–G) data. G1 through G5 indicates Groups 1 through 5. Each data is plotted and mean ± standard error of the mean was expressed as a set of the horizontal bar and error bars in each group. (A) Systolic arterial blood pressure in mm Hg at the end of experiment ($n=5$ for G1, 9 for G2, 12 for G3, 5 for G4 and 11 for G5). (B) Total cholesterol (mg/dl, $n=5$ for all groups). (C) Blood urea nitrogen (mg/dl, $n=5$ for all groups). (D) Creatinine (mg/dl, $n=5$ for all groups). (E) Rennin (ng/ml, $n=5$ except for G4 in which $n=4$). (F) Angiotensin II (pg/ml, $n=5$ except for G4 in which $n=4$). (G) Estradiol (pg/ml, $n=5$ except for G 2 and 5 in which $n=4$). Statistical analysis was performed to investigate differences between Group 1 vs. Group 2 or 3 by using one way analysis of variance followed by Dunnett's test or between Group 4 vs. Group 5 by using unpaired t-test. Significant statistical differences were expressed as $^{\#}P<0.05$ and $^{\#\#}P<0.01$.

groups (Table 1). Groups 1 and 4 without treatment served as the control S/D and Dahl rats, respectively ($n=5$ in each group). These control rats were sacrificed at a mean age of 9 weeks and used to confirm normal appearance of vascular corrosion cast described below and to obtain baseline blood sample data.

In groups 2, 3 and 5, the surgical procedures described in Table 1 were performed in one stage under anesthesia. These rats were given 1% NaCl in the drinking water beginning on the same day of the surgery and allowed to survive for 13–14 weeks and sacrificed at a mean age of 21 weeks. Nine of 18 rats treated as group 2, 12 of 16 group 3 rats and 11 of 15 group 5 rats survived this period and included for analysis of vascular corrosion casts and blood sample measurements, resulting in mortality rate of 50%, 25% and 31%, respectively.

Blood pressure was measured once a month in unanesthetized animals using the tail-cuff auto-pickup method (Softron Inc., Tokyo, Japan). Three months after the surgical procedure, the animals were anesthetized and blood samples (4 ml) were withdrawn, followed by vascular corrosion cast formation.

4.2. *Vascular corrosion cast formation*

Vascular corrosion casts were produced as reported previously (Jamous et al., 2005a; Jamous et al., 2005b; Reidy and Levesque, 1977). After induction of anesthesia, the rats underwent laparotomy and thoracotomy. The tip of a plastic cannula (18-gauge caliber with a length of 1.25 in.) was inserted into the left ventricle and secured in the ascending aorta with a superimposed ligature. After the right atrium was cut for drainage, the rats were perfused with 100 ml heparin/phosphate-buffered saline (20 U/ml) using a perfusion pump at 10 ml/min. This procedure was followed by manual injection of 10 ml Batson No. 17 plastic (Polysciences, Inc., Warrington, PA). After a 24-h period for polymerization at

Table 4 – Summary of experimental cerebral aneurysm model in Sprague–Dawley rats.

(1) Reports on branching site lesions

Reference	Age	Sex	Method of AN induction	Number of rats	Method of AN evaluation	Time from 1st surgery to sacrifice	Results [a]	
							Stage III	Stage I/II
Jamous et al., 2005b	7 W	F	bRA+uCCA+1 W+Salt+1 Mo+bOo	15	Cast + SEM	3 Mo	12 lesions in 9 (60%) rats	NE
Jamous et al., 2005c	7 W	F	bRA+uCCA+1 W+Salt+1 Mo+bOo	15	Cast + SEM	3 Mo	8 stage III lesions in 8 (53%) rats	5 stage I/II lesions in 5 (33%) rats
Present study (group 3)	7 W	F	bRA+uCCA+bOo+Salt (1 stage)	12	Cast + SEM	3 Mo	8 stage III lesions in 6 (50%) rats	12 stage I/II lesions in 8 (67%) rats

(2) Reports on non-branching site lesions

Reference	Age	Sex	Method of AN induction	Number of rats	Method of AN evaluation	Time from 1st surgery to sacrifice	Results on P1			
							Dilatation	Tortuosity	Fusiform AN	Lateral wall AN
Kondo et al., 1997	5 W	–	bRA+uCCA+1 W+Salt	35	PF+LM	12 Mo	31 (89%) rats showed one or both		22 ANs in 17 (49%) rats	8 ANs in 6 (17%) rats
Present study (group 3)	7 W	F	bRA+uCCA+bOo+Salt (1 stage)	12	Cast+SEM	3 Mo	11 (92%) rats showed one or both		1 AN in 1 (8%) rat	1 AN (lateral wall) in 1 (8%) rat

[a] See text for staging and type descriptions AN: aneurysm, bOo: bilateral oophorectomy, bRA: ligation of posterior branches of bilateral renal arteries, F: female, Mo: month, M: male, NE: not evaluated, P1: proximal segment of posterior cerebral artery, Salt: 1% salt in drinking water, SEM: scanning electron microscopy, uCCA: ligation of unilateral common carotid artery, W: week.

room temperature, the entire brain was removed and digested in 20% KOH for 24 to 72 h, with intermittent water rinses. The remaining vascular cast was mounted on the stage of a scanning electron microscope (model S-3200 N; Hitachi, Tokyo, Japan) using colloidal silver paste, sputter-coated with osmium, and screened for arterial abnormalities.

Hemodynamic stress is one of the most important factors in the induction of cerebral aneurysms, so we investigated the following sites of increased hemodynamic stress in the collateral circulation caused by right CCA occlusion: along the left ICA through the left ACA, along the basilar artery through the right PCA, and the right PcoA to the right ICA. The bilateral A1 segments form the azygos artery before separation into the bilateral distal ACAs, and there is no obvious AcoA in the rat (Lee, 1995). The relatively large OA arises from the A1 segment just before it merges into the azygos artery. The A1 segment is also characterized by frequent formation of fenestrations. The main flow into the PCA comes from the ICA through the PcoA, and the P1 that originates from the basilar terminal portion is relatively smaller than the PcoA in the rat.

4.3. *Classification of arterial changes according to scanning electron microscopy findings*

Resulting lesions were classified based on the scanning electron microscopy findings as defined previously for arterial branching sites (Jamous et al., 2005c) and for non-branching sites (Kondo et al., 1997) with some modifications. Rats have no so-called anterior communicating artery (AcoA) (Lee, 1995). However, many previous studies of experimental cerebral aneurysms have described lesions on the AcoA (Hashimoto et al., 1979a, 1979b; Nagata et al., 1980). We actually found fine branches connecting the proximal segment (A1) of the bilateral ACAs in both normal and dilated branches mimicking the AcoA in our experimental rats. Therefore, these branches have been termed the A1-communicating branches in this study. Almost all rats develop fenestrations in the A1 segment (Lee, 1995), which is one of the most common sites for development of aneurysmal changes in this rat model.

4.3.1. *Lesions at the branching sites*

(i). Stage 0 or normal: normal endothelial cell imprints at the arterial bifurcation with no gross arterial dilatation compared to the contralateral side (Fig. 1A).
(ii). Stage I: roughened apical intimal pad with irregularly shaped imprints (Fig. 1B).
(iii). Stage II: shallow fusiform elevation at the apical intimal pad covered with abnormal imprints (Fig. 1C).
(iv). Stage III: Saccular aneurysm covered with abnormal imprints (Fig. 1D).

4.3.2. *Lesions at the non-branching sites*

(i). Dilatation: for the proximal segment (P1) of the right posterior cerebral artery (PCA), uniform or multiple increases in the diameter of the artery along its longitudinal direction with maximum diameter ≥1.5 times that of the contralateral corresponding artery was considered to represent dilatation (Figs. 2A, C). For the A1-communicating branches, an increase in maximum diameter greater than that of the left OA was considered to represent dilatation (Fig. 2B). Beaded appearance was sometimes observed due to multiple dilatations along the right P1 or A1-communicating branches and was included in the category of dilatation.
(ii). Tortuosity: for the right P1 segment, at least 3 sharp curvatures (<45°) or a minimum of one loop was considered as tortuosity (Fig. 2C). For the A1-communicating branches, a hair pin-like curve caused by presumed longitudinal elongation was considered to represent tortuosity (Fig. 2B).
(iii). Fusiform aneurysm: Focal, segmental and circumscribed dilatation involving an entire vessel wall ≥1.5 times the diameter of the normal portion or the diameters at both ends was considered to represent fusiform aneurysm (Fig. 2D).
(iv). Lateral wall aneurysm: Saccular protrusion involving only a part of the vessel wall (Fig. 3).

4.4. *Laboratory analysis*

Blood samples for laboratory analysis were collected from 5 rats in each of the 5 groups. At the end of the 3-month period, after insertion of the cannula through the left ventricle into the ascending aorta, 4 ml of blood was withdrawn and mixed with ethylenediaminetetraacetic acid, centrifuged for serum isolation (5 min, 10.000 rpm), and stored in a refrigerator prior to examination. Cholesterol, creatinine, BUN, renin, angiotensin II, and estradiol levels were measured by the Special Reference Laboratory (SRL, Tokyo, Japan) in order to evaluate possible influence from the surgical procedures including renal artery occlusion and oophorectomy.

4.5. *Statistical analysis*

Two independent groups were compared by the unpaired t-test. Three or more independent groups were compared by one way analysis of variance with Dunnett's *post-hoc* test. Categorical data for groups were tested using the Chi-square test. Statistical analyses were performed on a computer running statistical software (GraphPad Prism v.5, San Diego, CA). Differences were considered statistically significant with a probability value of less than 0.05.

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Research Report

Effect of olmesartan and pravastatin on experimental cerebral aneurysms in rats

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ABSTRACT

The major initiation process of intracranial aneurysms is thought to involve endothelial dysfunction due to hemodynamic stress. Angiotensin II type 1 receptor blockers and statins improve vascular endothelium function. The effects of olmesartan and pravastatin were investigated on the development of experimental aneurysms in rats. Eighty-three rats underwent aneurysm induction. Seven groups of 10–14 rats were treated with low or high dose olmesartan, low or high dose pravastatin, low doses of olmesartan and pravastatin, hydralazine, or no drug (control) for 12 weeks, when rats were sacrificed for vascular corrosion casting and scanning electron microscopy. Aneurysmal changes at the anterior cerebral–olfactory artery bifurcation were divided into stages 0 (no abnormality) to III (saccular aneurysm). Systolic arterial blood pressure was elevated over 170 mm Hg in the control, low dose pravastatin, and high dose pravastatin groups, but not in the other groups. The control group demonstrated aneurysmal changes in 100% and stage III in 50% of rats. Aneurysmal changes were observed in most rats in the other groups, but the incidence of stage III was 10% or less. The staging pattern showed significant differences between the groups ($P=0.028$). Pravastatin reduced both stages III and II+III and olmesartan ameliorated stage III, implying that these may prevent aneurysmal formation through acting on different steps. (209 words)

1. Introduction

Subarachnoid hemorrhage, usually caused by rupture of an intracranial aneurysm, occurs in 10.5–23/100000 persons/year (Inagawa, 2001; Linn et al., 1996), and the population-based fatality rate of subarachnoid hemorrhage may be as high as 32–67% (Hop et al., 1997; van Gijn et al., 2007). Hemodynamic stress at the arterial wall of a bifurcation is one of the major

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Abbreviations: AC, anterior cerebral; ARB, angiotensin II type 1 receptor blocker; BUN, blood urea nitrogen; CCA, common carotid artery; eNOS, endothelial nitric oxide synthase; NADPH, nicotinamide adenine dinucleotide phosphate; NF-κB, nuclear factor κB; NO, nitric oxide; OA, olfactory artery; RAS, renin–angiotenisn system; ROS, reactive oxygen species; SABP, systolic arterial blood pressure; S/D, Sprague–Dawley

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factors in aneurysm development in humans and animals (Stehbens, 1989; Nagata, 1979). Most widely used experimental models of aneurysm employ induced hypertension and unilateral carotid artery ligation to induce the aneurysm, mainly at the contralateral anterior cerebral–olfactory artery bifurcation, which is the location of increased hemodynamic stress (Hashimoto et al., 1983; Hazama et al., 1986; Jamous et al., 2005a, 2007).

The initiation of aneurysm formation due to hemodynamic stress is associated with dysfunction of the arterial endothelium characterized by deficit of endothelial nitric oxide synthase (eNOS) next to the apical intimal pad (Meng et al., 2007; Jamous et al., 2005a). Various inflammation-related substances are involved in this early induction of cerebral aneurysm (Aoki et al., 2007b, 2009a; Tamura et al., 2009). Leukocyte and platelet adhesions are accelerated to further promote inflammation of the wall (Radomski et al., 1987; Kubes et al., 1991; Aoki et al., 2007a). Smooth muscle cell (SMC) migration then causes thinning of this part of the arterial wall and outwards expansion. In addition, proteinases such as matrix metalloproteinase (MMP)-1, -2, and -9 and tissue plasminogen activator are upregulated and cause excessive degradation of the extracellular matrix (Bruno, 1998; Aoki et al., 2007a).

Angiotensin II type 1 receptor blockers (ARB) and hydroxymethylglutaryl-coenzyme A reductase inhibitors (statins) are widely used anti-hypertensive and anti-hypercholesterolemic agents, respectively. Both also have pleiotropic effects, such as improving vascular endothelium function and suppressing inflammation and oxidative stress in animals (Kumai et al., 2008; Ito et al., 2002; Vasa et al., 2001). Therefore, these agents may have protective effects against aneurysm development, but little is known except for statins (Aoki et al., 2008).

The present study investigated the effect of olmesartan, an ARB, and pravastatin, a statin, on the development of cerebral aneurysms in the rat model.

2. Results

2.1. *Blood pressure at the experiment end*

The experimental groups are explained in Table 1. Renal artery occlusion induced hypertension >170 mm Hg systolic arterial blood pressure (SABP) in control (Group C), low dose pravastatin (Group PL), and high dose pravastatin groups (Group PH) with no statistical difference (Table 1). Blood pressure remained moderate at around 137–150 mm Hg SABP in hydralazine (Group H), low dose olmesartan (Group OL), high dose olmesartan (Group OH), and mixed (low dose pravastatin + low dose olmesartan) groups (Group M) with no statistical difference. The mean SABPs in Groups OL, OH, and M was significantly different from that of Group C ($P<0.05$).

Mortality rate during the experiment was 7% (1/15) in Group C, 8% (1/12) in Group H, 17% (2/12) in Group PL, and 0% in other groups.

2.2. *Classification of arterial changes according to scanning electron microscopy*

The most susceptible site for aneurysmal formation in this model is the anterior cerebral–olfactory artery bifurcation contralateral to the side of common carotid artery ligation. These lesions were classified in 4 stages as shown in Fig. 1, based on the scanning electron microscopy findings as defined previously for arterial branching sites (Jamous et al., 2005a,b; Eldawoody et al., 2009).

Table 1 – Experimental groups and blood pressure at the end of the experiment.

Group	Treatment	Number of rats	SABP (mm Hg)
C	1% saline	14	170±22
H	1% saline + Hydralazine 3 mg/kg/day	11	150±16
OL	1% saline + Olmesartan 3 mg/kg/day	13	146±14#
OH	1% saline + Olmesartan 10 mg/kg/day	12	141±14#
PL	1% saline + Pravastatin 50 mg/kg/day	10	186±29
PH	1% saline + Pravastatin 100 mg/kg/day	12	175±24
M	1% saline + Olmesartan 3 mg/kg/day + Pravastatin 50 mg/kg/day	11	137±17#

C, control (1% saline); H, hydralazine; OL, low dose olmesartan; OH, high dose olmesartan; PL, low dose pravastatin; PH, high dose pravastatin; M, mixed (low dose pravastatin + low dose olmesartan); SABP, systolic arterial blood pressure at the end of the experiment (mean ± standard error of the mean).
#Statistically significant difference in SABP was detected against C group by one way analysis of variance with Dunnet's post-hoc test. ($P<0.0001$).

Resulting aneurysmal changes at the anterior cerebral–olfactory artery bifurcation are summarized in Table 2 and Figs. 2A–C. Total number of aneurysms was not significantly different between the groups. The pattern of staging showed statistically significant differences between the groups ($P=0.028$, Chi-square test).

Further comparison of each group against Group C was conducted as demonstrated in Figs. 2A–C. Fig. 2A summarizes the effect of olmesartan in comparison to Groups C and H disclosing that the staging pattern of Groups H and OH was statistically different from that of Group C ($P=0.03$ and 0.02, respectively). The pattern of Group OL tended to be different from that of Group C, but the difference was not statistically significant ($P=0.06$).

Fig. 2B summarizes the effect of pravastatin in comparison to Group C. The staging pattern of Groups PL and PH was statistically different from that of Group C ($P=0.01$ and 0.03, respectively).

Fig. 2C indicates that Group M, which is a combination of OL and PL, was significantly different from Group C ($P=0.01$) but failed to demonstrate an additive effect over Groups OL and PL.

No significant difference was found between Groups OL and OH, and between Groups PL and PH, so combined groups of OL + OH and PL + PH were compared with Groups C, H, and M. Chi-square testing of these five groups yielded a P value of 0.003. Comparison between each group and Group C showed significant differences in C vs. OL + OH ($P=0.006$), C vs. PL + PH

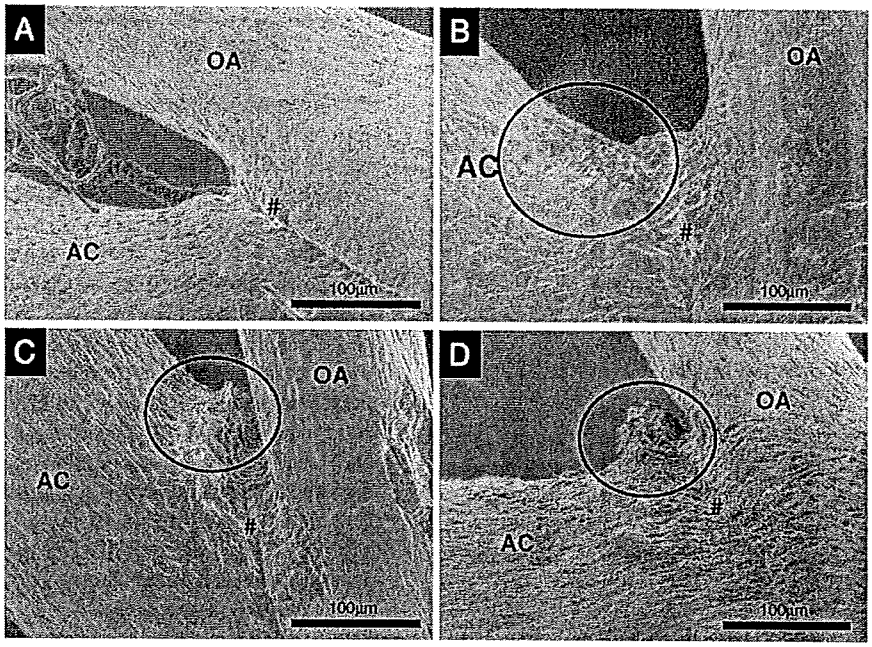


Fig. 1 – Classification of aneurysmal changes at the anterior cerebral–olfactory artery bifurcation contralateral to the side of common carotid artery ligation (Jamous et al., 2005a,b; Eldawoody et al., 2009). Vascular corrosion casts were observed under the scanning electron microscope. (A) Stage 0 (Normal): Normal endothelial cell imprints at the arterial bifurcation with no gross arterial dilatation compared to the contralateral side. (B) Stage I: Roughened apical intimal pad with irregularly shaped imprints. (C) Stage II: Shallow fusiform elevation at the apical intimal pad covered with abnormal imprints. (D) Stage III: Saccular aneurysm covered with abnormal imprints.

(P=0.013), and OL+OH vs. PL+PH (P=0.024). No differences were demonstrated in H vs. OL+OH (P=0.842), M vs. OL+OH (P=0.334), and M vs. PL+PH (P=0.874).

Table 2 – Number of induced aneurysmal changes at the left anterior cerebral–olfactory artery bifurcation in each group.

Group	Number of rats	No lesion (%)	Stage I (%)	Stage II (%)	Stage III (%)	Total number of aneurysms (%)
C	14	0 (0%)	3 (21%)	4 (29%)	7 (50%)	14 (100%)
H	11	1 (9%)	4 (36%)	5 (45%)	1 (9%)	10 (91%)
OL	13	1 (8%)	3 (23%)	8 (62%)	1 (8%)	12 (92%)
OH	12	1 (8%)	4 (33%)	7 (58%)	0 (0%)	11 (92%)
PL	10	1 (10%)	6 (60%)	2 (20%)	1 (10%)	9 (90%)
PH	12	3 (25%)	6 (50%)	2 (17%)	1 (8%)	9 (75%)
M	11	2 (18%)	5 (45%)	3 (27%)	1 (9%)	9 (82%)

Statistically significant difference in the staging pattern was detected between groups (P=0.028, Chi-square test). Further comparison of each group against Group C disclosed that the staging pattern of Groups OH and PH were statistically different from that of Group C (P=0.031 and 0.028, respectively).

2.3. *Blood sample measurements*

Figs. 3A–C summarize blood sample measurements obtained at the end of the experiment, arranged to be comparable with Figs. 2A–C. Group N indicates data from three age-matched normal S/D rats (20-week-old).

In Fig. 3A representing the effect of olmesartan, total cholesterol showed weak but significant differences among 5 groups and Group OH showed statistically significant difference against Group N by Bonferroni post-hoc test for multiple comparison. Renin and angiotensin II appeared to be reduced in Groups C and H, but there was no statistical significance probably due to a small sample number with a large variation in Group N. Sodium (Na) and potassium (K) showed significant differences among 5 groups, but the Bonferroni test revealed statistically significant difference only between Groups N and OH. Estradiol levels appeared to be reduced in experimental Groups compared with the mean value reported in our previous report (Eldawoody et al., 2009), although no statistical analysis was performed.

In Fig. 3B representing the effect of pravastatin, total cholesterol showed a statistically significant but weak difference among 4 groups. Renin and angiotensin II appeared to be reduced in experimental Groups, but there was a statistical significance only for renin. Groups C and PL were significantly different from Group N, respectively. Sodium (Na) and potassium (K) did not show significant differences among 5 groups. Reduction in estradiol levels of Groups PL and PH appeared to be less profound compared to the normal value and Group C.

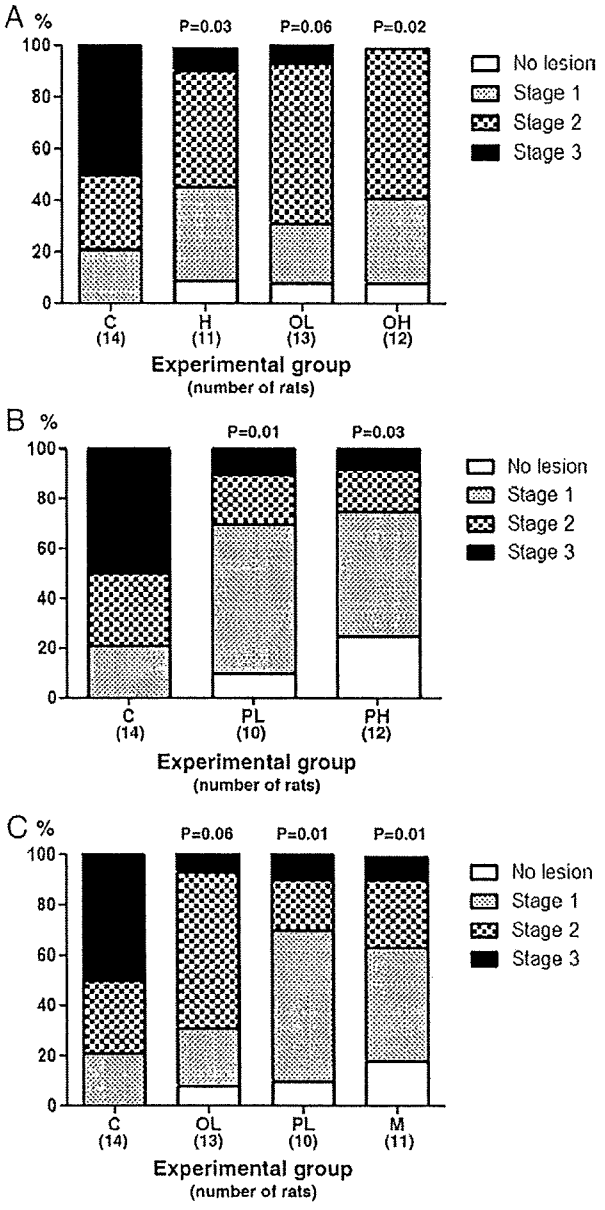


Fig. 2 – Aneurysmal changes observed at anterior cerebral–olfactory artery bifurcation in each group. A: a graph showing the effect of olmesartan in comparison to Groups C and H. The staging patterns of Groups H and OH were statistically different from that of Group C (P=0.03 and 0.02, respectively). Difference between Groups OL and C did not reach statistical significance (P=0.06). B: a graph showing the effect of pravastatin in comparison to Group C. The staging patterns of Groups PL and PH were statistically different from that of Group C (P=0.01 and 0.03, respectively). C: a graph showing that Group M (combination of OL and PL) was significantly different from Group C (P=0.01) but not from Groups OL and PL.

Fig. 3C indicates that Group M, which is a combination of OL and PL, was not significantly different from Group N, OL or PL in any of the measured parameters.

3. Discussion

This study demonstrated that neither olmesartan nor pravastatin affects the total number of induced aneurysms, but both agents reduced development of stage III aneurysms. Stage III aneurysms were detected in 50% of Group C, but in only 0–10% in groups treated with olmesartan and/or pravastatin.

In Fig. 2A showing the effect of olmesartan, the decrease in stages III aneurysms may be dose-dependent, although not proved statistically. In contrast, olmesartan did not reduce combined stages II+III, suggesting that olmesartan worked to prevent the progression from stage II to III. On the other hand, pravastatin reduced aneurysms of not only stage III but also combined stages II+III. Together with no reduction in stage I and total number of aneurysms, main effect of pravastatin may be through prevention of the progression from stage I to II, with additional effect on the process from stage II to III (Fig. 2B). These data at least suggest that olmesartan and pravastatin exerted the effects in different stages of aneurysmal formation, respectively.

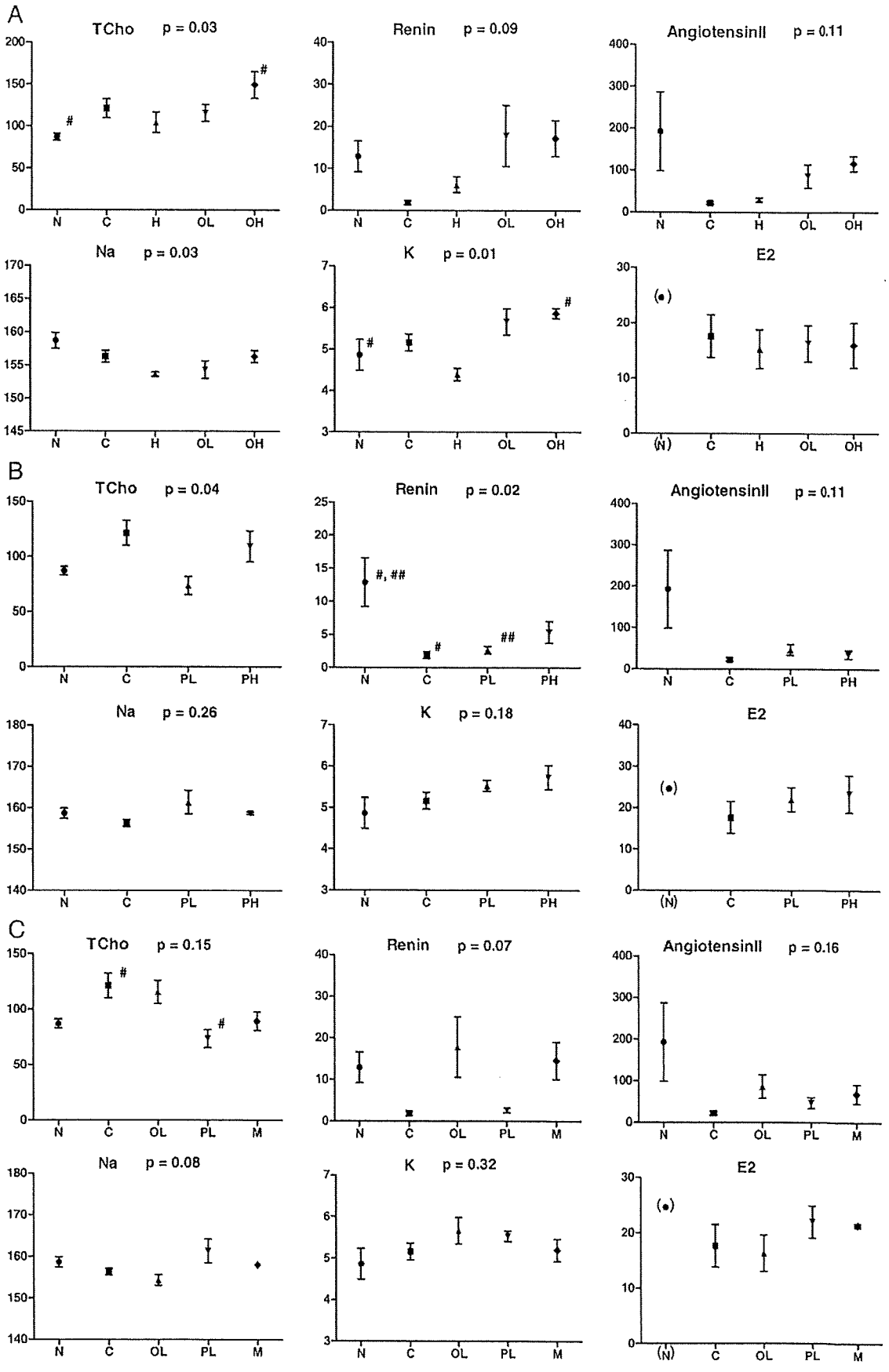
3.1. *Mechanism of development of cerebral aneurysms*

Hemodynamic stress at the arterial wall of the bifurcation is one of major factors of aneurysm development in humans and animals (Stehbens, 1989; Nagata, 1979). Blood flow impinges on the arterial bifurcation and dynamic changes in wall shear stress occur at the apex (Meng et al., 2007), leading to endothelial cell dysfunction which is characterized by reduction of expression of eNOS. Nitric oxide (NO) generated by eNOS attenuates vasoconstriction, platelet aggregation, and expression of various adhesion molecules as well as reactive oxygen species (ROS). Normal vascular function is maintained by the balance between NO and ROS. The imbalance (NO<ROS) due to hemodynamic stress causes vascular inflammation associated with upregulation of monocyte chemoattractant protein-1, vascular cell adhesion molecule-1, and other mediators of inflammatory cell adhesion to the vascular wall (Marui et al., 1993; De Keulenaer et al., 2000).

Nuclear factor κB, a family of transcriptional factors regulating gene expression related to inflammatory mediators, is thought to be central in the early changes of such inflammation (Pahl, 1999). Macrophages infiltrating into vascular wall produce MMP-2 and -9 which degrade the extracellular matrix of the arterial wall (Aoki et al., 2007a). Macrophages also contain nicotinamide adenine dinucleotide phosphate oxidase which is one of the major enzymes for ROS production. Thus, a positive cascade of vascular inflammation is developed. Together with apoptosis of the vascular SMCs (Kondo et al., 1998), these inflammatory reactions are thought to be essential to cerebral aneurysm formation (Aoki et al., 2007b, 2009c).

3.2. *Effect of ARB on cerebral aneurysm development*

Angiotensin II is a potent pro-inflammatory agent that acts through the angiotensin II type 1 receptor. Angiotensin II-induced increase of ROS through nicotinamide adenine dinucleotide phosphate oxidase activation is key in the


A
TCho p = 0.03
Renin p = 0.09
AngiotensinII p = 0.11
Na p = 0.03
K p = 0.01
E2
N C H OL OH
B
TCho p = 0.04
Renin p = 0.02
AngiotensinII p = 0.11
Na p = 0.26
K p = 0.18
E2
N C PL PH
C
TCho p = 0.15
Renin p = 0.07
AngiotensinII p = 0.16
Na p = 0.08
K p = 0.32
E2
N C OL PL M

connection between angiotensin II and the inflammatory process described above (Marui et al., 1993; De Keulenaer et al., 2000; Chen et al., 1998; Tummala et al., 1999; Cai and Harrison, 2000). Thus, activation of renin–angiotensin system (RAS) in the vascular wall enhances these inflammatory processes and causes arteriosclerosis (Diet et al., 1996; Ruiz-Ortega, 2001).

Even a small amount of ARB can suppress such oxidative stress in animals (Kumai et al., 2008; Ito et al., 2002). ARB can prevent abdominal aortic aneurysm, even at a dosage not sufficient for lowering blood pressure (Fujiwara et al., 2008). This mechanism is thought to depend on the prevention of accumulation of macrophages into the vessel wall (Fujiwara et al., 2008).

The present results suggest that ARB can prevent the development of aneurysm compared to Group C. At the same time, Group H showed reduction in aneurysmal induction similar to the olmesartan groups. The effect of olmesartan may be due to the anti-hypertensive effect rather than the pleiotropic effects of ARB in a large portion. However, there was a decreasing tendency of stage III aneurysms in Group OH compared with Group H (Fig. 2A), so possible contribution of the pleiotropic effects cannot be ruled out. The importance of RAS in cerebral aneurysm formation is controversial compared to well-known roles in aortic aneurysms or other vascular diseases. While it is reported that RAS might be less important in cerebral aneurysm formation compared to aortic aneurysm or other vascular diseases (Aoki et al., 2009d), local RAS activation increased the formation and progression of cerebral aneurysm and its inhibition reduced the aneurysmal changes without lowering blood pressure (Tada et al., 2009). To discriminate the pleiotropic effects from the blood pressure lowering effect, other approaches, such as using very low dose ARB with minimum effect on the blood pressure, blood pressure elevating agents together with ARB or use of an antioxidant for comparison, will be necessary.

Fig. 3A indicates that total cholesterol increased in Group OH in comparison to Group N. The reason of this cannot be fully clarified and further investigation will be necessary to draw a conclusion. Tendency of reduction in renin and angiotensin II in Group C may be a result of renal infarction produced surgically in this animal model. Increasing tendency of renin and angiotensin II in Groups OL and OH may be due to a positive feedback to increase the renin and angiotensin II levels following a block of residual angiotensin II. There was a weak reduction in sodium concentration and a significant elevation in potassium concentration following olmesartan treatment, which are known side effects of ARB.

3.3. *Effect of statin on cerebral aneurysm development*

Statins also have pleiotropic effects that protect vascular endothelium from hemodynamic and oxidative stress. Statins stimulate the phosphoinositide-3 kinase/Akt process and increase NO activity through eNOS upregulation (Vasa et al., 2001). Statins also attenuate vascular remodeling processes by inhibiting expression of MMPs by SMCs and macrophages (Hayashidani et al., 2002). MMPs may be very important in extracellular matrix degradation, so inhibition may reduce the arterial wall thinning possibly involved in aneurysm formation.

Simvastatin attenuates the induction of cerebral aneurysm through suppression of vascular inflammation processes (Aoki et al., 2008). Statins may also inhibit thinning of SMCs by modulating the expression of interleukin-1beta and inducible NOS, which are known to induce apoptosis of SMCs (Moriwaki et al., 2006; Fukuda et al., 2000; Sadamasa et al., 2003). Pitavastatin also suppresses the development of cerebral aneurysms associated with inhibition of nuclear factor-κB activation and apoptotic cell death (Aoki et al., 2009b).

The present study indicated that pravastatin also exhibited a protective effect against formation of cerebral aneurysms. Statin treatment significantly reduced the development of stage III and II+III aneurysms. Although total aneurysmal changes were only mildly reduced, number of rats without aneurismal lesions tended to increase in a dose-dependent manner (Fig. 2B). It will be expected to investigate what stages of aneurismal formation statin works for.

Fig. 3B indicates that total cholesterol did not decrease significantly in Groups PL and PH in comparison to Groups N and C. The reason of this may be due to lower or normal baseline total cholesterol levels in rats used for the experiment together with a small sample size. Reduction in renin and such tendency in angiotensin II in Groups PL and PH may suggest that statin has little effect on the renin–angiotensin system.

3.4. *Combination of ARB and statin*

Combination of ARB and statin is reported to have a stronger effect in preventing the progression of aortic aneurysms

Fig. 3 – Results of blood sample analysis. Arterial blood samples were obtained from 3 rats in each group at the end of the experiment. Group N indicates another set of three normal female S/D rats of 20-week-old. The normal value of estradiol was cited from our previous report (Eldawoody et al., 2009) and presented with parenthesis. A: graphs representing the effect of olmesartan. Total cholesterol (TCho) showed weak but significant differences among 5 groups (P=0.03) and Group OH showed statistically significant difference against Group N (Bonferroni post-hoc test for multiple comparison). In renin and angiotensin II, there was no statistically significant difference. Sodium (Na) and potassium (K) showed significant differences among 5 groups with positive Bonferroni test between Groups N and OH in K data. Estradiol levels appeared to be reduced in experimental Groups compared with the mean value reported in our previous report (Eldawoody et al., 2009), although no statistical analysis was performed. B: graphs representing the effect of pravastatin. TCho showed a statistically significant but weak difference among 4 groups. Renin and angiotensin II appeared to be reduced in experimental Groups, but there was a statistical significance only for renin. Groups C and PL were significantly different from Group N, respectively. Sodium (Na) and potassium (K) did not show significant differences among 5 groups. Reduction in estradiol levels of Groups PL and PH appeared to be less profound compared to the normal value and Group C. C: graphs indicating that mean values of Group M tended to be intermediate between Groups OL and PL and not significantly different from these groups in any of the measured parameters.

associated with improved eNOS activity. This additive effect probably depends on the different pathways of ARBs and statins to restore or activate eNOS. Olmesartan suppresses eNOS dimer disruption through amelioration of ROS, whereas pravastatin enhances the phosphorylation of Akt which is known to enhance eNOS activity (Imanishi et al., 2008; Yamamoto et al., 2007; Davignon, 2004). Fig. 3C demonstrates that most of the values of Group M were between Groups OL and PL, suggesting additive effects, however, such effects were not evident in terms of aneurysmal formation shown in Fig. 2C. No conclusive statement can be made before it is confirmed in further investigations employing different approaches mentioned in the Section 3.2.

3.5. *Limitations of the present study and conclusion*

One of the important limitations of this study may be a lack of measurement of actual dose of the drugs that each rat took. Measurement of blood concentration of olmesartan and pravastatin would have been ideal, which was not possible in this particular study. Changes in SABP may partially and indirectly imply active intake of drugs (Table 1). We also introduced previous studies which indicate rough estimates of daily amount of food and water intake of a rat (Aoki et al., 2008; Cruzan, 2009).

Other than the issue of blood pressure in groups administered ARB, another limitation of this study includes relatively small number of animals in each group. Especially, the blood sample analysis was performed in three rats to obtain rough outline of the parameters and should be considered as pilot data.

In conclusion, treatments with olmesartan and pravastatin suppressed the development of experimental aneurysms in rats. The steps and/or mechanisms of aneurysmal development affected by both agents seem to be different and remain to be investigated further.

4. Experimental procedures

4.1. *Experimental models and groups*

A total of 83 seven-week-old female Sprague–Dawley rats (Hamri Co. Ltd., Ibaraki, Japan) weighing between 150 and 200 g were kept under optimum conditions (temperature 23 ±3 °C, humidity 50±20%) in the Institute for Animal Experimentation, Tohoku University Graduate School of Medicine, throughout the experiment. The experiments and protocols were conducted in accordance with the Japanese standards for the care and use of laboratory animals and were approved by the Animal Care Committee, Tohoku University Graduate School of Medicine. All surgical procedures were performed after induction of anesthesia by intraperitoneal injection of pentobarbital 40 mg/kg.

Our surgical procedures were based on previous reports (Jamous et al., 2005a,b), but differed significantly in performing all surgical procedures in one stage (Eldawoody et al., 2009). After anesthesia and midline laparotomy, ligation of posterior branches of bilateral renal arteries and bilateral oophorectomy were performed. The right common carotid artery was also ligated. All rats were given 1% NaCl in the drinking water beginning on the same day as surgery.

The rats were divided into 7 groups according to the post-surgical treatments as shown in Table 1. Olmesartan medoxomil (Daiichi-Sankyo Co., Tokyo, Japan) was dissolved into 0.1% $NaHCO_3$–0.1% $KHCO_3$ solution and administered in the drinking water. The doses for Groups OL and OH were based on the previous report (Koike et al., 2001). Daily water consumption of female S/D rats aged between 8 and 20 weeks (250–400 g) is around 35–40 ml (Cruzan, 2009). Considering some amount of spill, if approximately 80 ml/kg/day of water was actually taken by the rat, 0.0125 and 0.00375% olmesartan solution given to rats through a drinking bottle would correspond to giving 10 and 3 mg/kg/day, respectively. We expected olmesartan to reduce the blood pressure, so Group H (hydralazine 3 mg/kg in the drinking water) was included to discriminate the possible pleiotropic effects of olmesartan from the anti-hypertensive effect. Pravastatin sodium (Daiichi-Sankyo Co.) was administered in the diet. The doses were decided by referring the simvastatin study in a rat cerebral aneurysm model (Aoki et al., 2008). The study reported that an average daily food consumption of a rat is 25 g/day and that 25 mg/kg of oral administration of simvastatin was effective to demonstrate reduction in aneurysmal formation. Because 25 mg/kg of simvastatin is comparable to 50 mg/kg of pravastatin, we set this dose for Group PL and 100 mg/kg for Group PH.

4.2. *Blood pressure and blood sample measurements*

Blood pressure was measured once a month in the unanesthetized and calm condition by using the tail-cuff autopickup method (Softron Inc., Tokyo, Japan). Three months after the surgical procedure, the animal was anesthetized and blood samples were withdrawn for laboratory analysis from 3 rats in each group. After insertion of a plastic cannula (18-gauge caliber, length 1.25 in) through the left ventricle into the ascending aorta, 4 ml of blood was withdrawn and mixed with ethylenediaminetetra-acetic acid, followed by centrifugation for serum isolation (5 min, 10000 r/m), and storage in a freezer at −80 °C prior to analysis. The following serum parameters were analyzed by the Special Reference Laboratory (Tokyo, Japan): total cholesterol (Tcho), renin, angiotensin II, sodium (Na), potassium (K) and estradiol.

For comparison, another group of 3 female S/D rats (20-week-old) was used to measure baseline values of these parameters except estradiol. For normal estradiol data, we referred to our previous study which was conducted in parallel with the present study (Eldawoody et al., 2009).

4.3. *Vascular corrosion cast formation and classification of aneurysmal lesions*

Vascular corrosion casts were prepared as described previously (Jamous et al., 2005a,b; Reidy and Levesque, 1977; Eldawoody et al., 2009) after collection of the blood sample. In brief, the right atrium was cut for drainage and the rats were perfused with 100 ml heparin/phosphate-buffered saline (20 U/ml) using a perfusion pump at 10 ml/min. This procedure was followed by manual injection of 10 ml Batson No. 17 plastic (Polysciences, Inc., Warrington, PA). After a 24-hour period for polymerization at room temperature, the entire brain was removed and digested in 20% KOH for 24 to 72 h, with intermittent water rinses. The