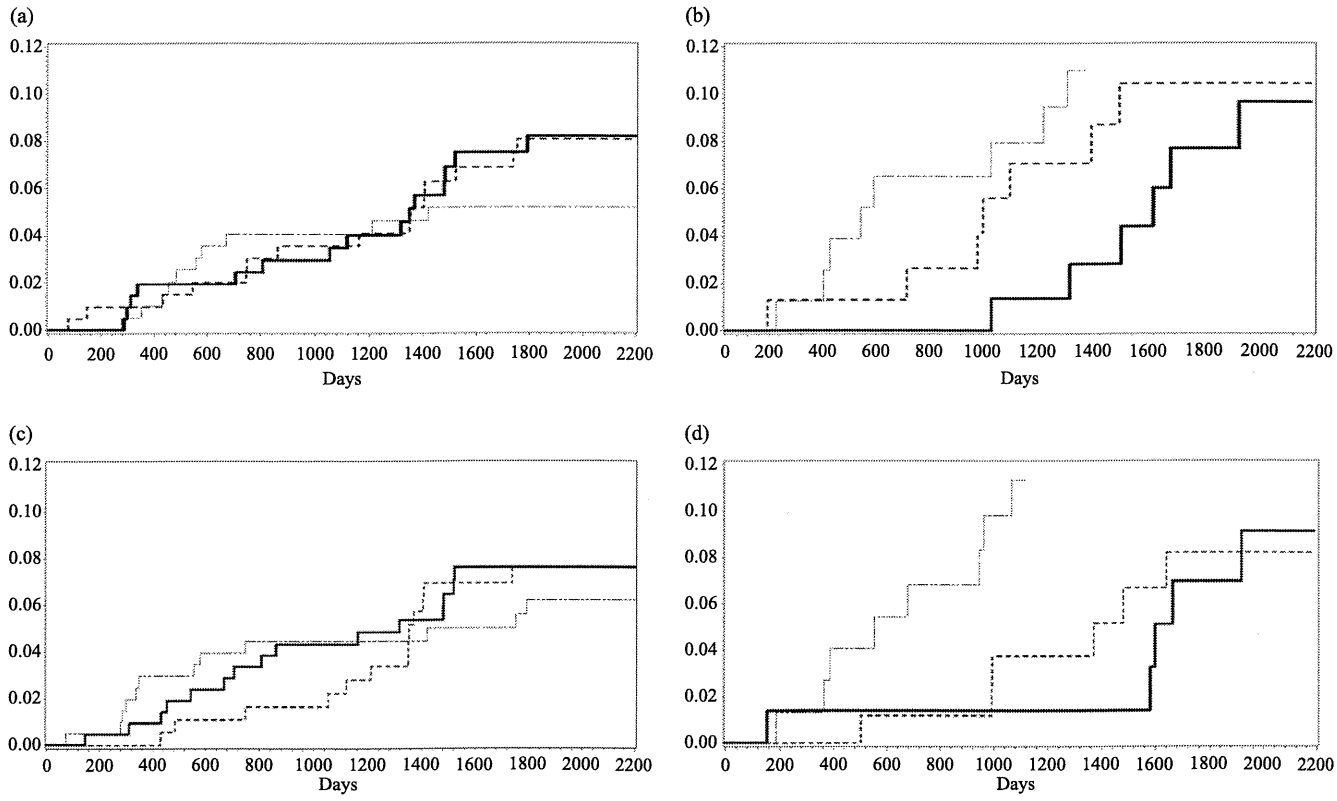

Figure 3 Kaplan–Meier curve for vegetable intake, fish intake and overall mortality. (a) Vegetable intake, young-old. b) Vegetable intake, old-old. (c) Fish intake, young-old. (d) Fish intake, old-old. Lower mortality rate was observed in old-old if vegetable intake was high. Also, when the fish intake was high, mortality rate was significantly low ($P = 0.0195$). Hazard rates for old-old to the amount of fish intake were 0.34 ($P = 0.0352$) for the group of medium amount intake, and 0.429 ($P = 0.117$) for high intake.

Table 5 Food Intake and all death

	Intake					
	Vegetable			Fish		
	P-value	Hazard ratio	CI	*P*-value	Hazard ratio	CI
All						
Log-rank	0.97	–	–	0.48	–	–
Middle intake (*vs* low intake)	0.86	1.06	(0.58–1.91)	0.29	0.72	(0.40–1.31)
High intake (*vs* low intake)	0.89	1.05	(0.57–1.91)	0.50	0.82	(0.46–1.47)
Young-old						
Log-rank	0.55	–	–	0.87		–
Middle intake (*vs* low intake)	0.34	1.49	(0.66–3.34)	0.70	1.17	(0.53–2.58)
High intake (*vs* low intake)	0.24	1.62	(0.73–3.63)	0.50	1.30	(0.61–2.77)
Old-old						
Log-rank	0.24	–	–	**0.0195***	–	–
Middle intake (*vs* low intake)	0.25	0.58	(0.22–1.48)	**0.0352***	**0.35**	**(0.13–0.93)**
High intake (*vs* low intake)	0.24	0.55	(0.20–1.49)	0.12	0.43	(0.15–1.24)

*P-value < 0.05.

We compared the background data for each dietary pattern, but there was no clear difference in the factors that possibly influence when comparing the pathogenesis of events.

As for the events that occurred in each dietary pattern, when all including young-old and old-old were subjected, statistically significant results were not obtained; however, we observed the tendency for more

deaths to occur in the greasy type and less in the healthy type in both analyses for overall mortality and diabetes-related deaths (Fig. 1).

From the Cox regression analyses adjusted by several factors, age was the highly significant factor. Considering the fact that the participants of the present study were a group of "elderly diabetes patients," we divided the subjects into young-old and old-old, and a Kaplan–Meier curve was drawn for overall mortality. As a result, the mortality rate for the greasy type and healthy type were almost equivalent, and higher in the snack type in young-old; however, in old-old, a higher mortality rate was reported in the greasy type, but a lower tendency was notable in the healthy type.

These findings suggested that the greasy type diet that includes a large amount of sugars, fats and meats is a factor for poor life prognosis, and the healthy type diet for elderly, especially for old-old, might reduce the occurrence of all deaths and diabetes-related deaths.

As the mechanism for these phenomena, we consider that with the greasy type dietary pattern, the risk of causing poor lipid metabolism, glucose metabolism and increased obesity, and aggravating arteriosclerosis are increased. In contrast, with the healthy type dietary pattern these would be improved or decreased.[17–19] However, J-EDIT subjects are Japanese and their BMI were not inherently very high. As shown in Table 2, the BMI are close to 24 in all dietary patterns. Therefore, we consider BMI was not the major contributor for the improved prognosis. Being diabetes patients of advanced age originally meant they were, in a way, long-term survivors. In particular, the old-old are long-term survivors and likely to be long-term patients of diabetes. However, taking these facts into account, the dietary patterns and mortality rates still showed a certain amount of tendencies. Therefore, we think dietary habit is of importance as one of the factors that determine life prognosis.

The characteristics of the healthy type dietary pattern are high intake of vegetables and seaweeds, and relatively high intake of fish. The analyses of vegetable and fish intake by tertiles (Fig. 3, Table 5) suggests that elderly, especially old-old, showed an inverse relationship of increased vegetables and fish consumption, and mortality pattern. This result is in agreement with previously reported studies.[20–22] This finding also strongly agrees with the report by Takahashi *et al.* published in the present issue of the journal, that concluded that an adequate amount of vegetable intake might lead to favorable control of blood glucose or triglyceride values.[23]

The reason for the relationship between the healthy type dietary pattern and better prognosis observed in old-old is not clear at this moment. Being able to eat an adequate amount of vegetables means the person retains healthy mastication ability. Such reversal of cause and effect might be considered as well. Also, the association of activated immune functions by vegetables,[24] an idea that is the current subject of active discussion, might be a possibility.

We have extracted three dietary patterns (healthy type, snack type and greasy type) from the diet survey of J-EDIT and life prognosis for the greasy dietary pattern with high intake of sugars, fats and meats is poor. In contrast, the patients in the healthy type dietary pattern group with a high intake of vegetables and fish will likely become the long-term survivors. The healthy type dietary pattern, with a high intake of vegetables and fish, has many similarities with the Mediterranean diet and DASH diet, and tends to have a lower number of death events occur compared with other types. Thus, the present results show the influence of dietary pattern on life prognosis cannot be neglected in elderly Japanese patients with type 2 diabetes.

The major limitation of the present study was the small number of subjects. Previous studies showing the importance of dietary patterns scaled the numbers of subjects in the tens of thousands to hundreds of thousands. The reasons we consider for not being able to obtain significance in the results of the present study are that this was a group of elderly patients, and the small size of the study; with fewer than 1000 patients, when limited to the old-old, the numbers of cases was 258. Estimation for the number of subjects to detect a statistically significant relationship between diabetes-related deaths and dietary pattern based on the data from the present study is as follows: assuming that diabetes-related death occurs in 1% in 1 year in the healthy type dietary pattern, the clinically significant hazard ratio of the greasy type dietary pattern is 1.25, and 6-year follow up is expected as in the present study, therefore a minimum of 15 000 cases is necessary. We hope that such a study will be carried out.

Acknowledgments

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Conflict of interest

The J-EDIT Study Group has not cleared any potential conflicts.

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Testosterone Deficiency Accelerates Neuronal and Vascular Aging of SAMP8 Mice: Protective Role of eNOS and SIRT1

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Abstract

Oxidative stress and atherosclerosis-related vascular disorders are risk factors for cognitive decline with aging. In a small clinical study in men, testosterone improved cognitive function; however, it is unknown how testosterone ameliorates the pathogenesis of cognitive decline with aging. Here, we investigated whether the cognitive decline in senescence-accelerated mouse prone 8 (SAMP8), which exhibits cognitive impairment and hypogonadism, could be reversed by testosterone, and the mechanism by which testosterone inhibits cognitive decline. We found that treatment with testosterone ameliorated cognitive function and inhibited senescence of hippocampal vascular endothelial cells of SAMP8. Notably, SAMP8 showed enhancement of oxidative stress in the hippocampus. We observed that an NAD^+-dependent deacetylase, SIRT1, played an important role in the protective effect of testosterone against oxidative stress-induced endothelial senescence. Testosterone increased eNOS activity and subsequently induced SIRT1 expression. SIRT1 inhibited endothelial senescence via up-regulation of eNOS. Finally, we showed, using co-culture system, that senescent endothelial cells promoted neuronal senescence through humoral factors. Our results suggest a critical role of testosterone and SIRT1 in the prevention of vascular and neuronal aging.

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Introduction

Advancing age is the most significant risk factor for the development of cognitive impairment [1,2]; however, what age-related changes underlie this effect remains uncertain. With advancing age, men experience a significant decrease in the circulating level of testosterone. Although studies have shown alterations in mood, libido, and cognition resulting from testosterone deficiency [3], the full range of consequences of age-related testosterone loss remains incompletely defined. In a small clinical study of men recently diagnosed with cognitive impairment, testosterone treatment improved performance on cognitive tests [4]. In a prospective longitudinal study using subjects from the Baltimore Longitudinal Study on Aging, men who developed Alzheimer disease (AD) were observed to exhibit low testosterone levels 5–10 years prior to the clinical diagnosis of AD [5]. With a relationship between age-related testosterone decline in men and increased risk for cognitive impairment reasonably well established, a critical issue is how testosterone contributes to the pathogenesis of cognitive decline with aging. The most likely hypothesis is through the regulation of accumulation of amyloid ß (Aß) peptides, which are widely believed to be the critical initiating step in the pathogenesis of AD. However, it is becoming increasingly clear that not all aspects of cognitive decline can be explained by Aß [6,7]. Findings from such diverse lines of investigations as neuroimaging and clinical trials suggest that non-Aß factors also contribute to memory deficit in aged men.

In *S. cerevisiae*, the *Sir2* (silent information regulator-2) family of genes governs budding exhaustion and replicative life span [8,9]. *Sir2* has been identified as an NAD^+-dependent histone deacetylase and is responsible for maintenance of chromatin silencing and genome stability. Mammalian sirtuin 1 (*Sirt1*), the closest homolog of *Sir2*, regulates the cell cycle, senescence, apoptosis and metabolism, by interacting with a number of molecules such as p53. As recently reported, overexpression of SIRT1 in the brain improved the memory deficit in a mouse model of AD via activation of the transcription of α-secretase [10].

An increasing body of evidence suggests the presence of a link between cognitive decline and vascular dysfunction, especially atherosclerosis [11]. Senescence of endothelial cells is involved in endothelial dysfunction and atherogenesis, and SIRT1 has been recognized as a key regulator of vascular endothelial homeostasis, controlling angiogenesis, endothelial senescence, and dysfunction [12–14].

In the present study, we demonstrated that cognitive impairment in senescence-accelerated mouse prone 8 (SAMP8), a model of cognitive decline with aging, is associated with endothelial senescence in the hippocampus and is ameliorated by testosterone

replacement. SIRT1 plays an important role in prevention of endothelial senescence induced by oxidative stress [13]. We suggest that the protection against endothelial senescence in the hippocampus through up-regulation of testosterone and SIRT1 could contribute to a novel therapeutic strategy against cognitive decline with aging.

Results

Treatment with dihydrotestosterone ameliorated cognitive function of SAMP8

In order to assess the effects of testosterone on cognitive function, we used an in vivo model of aging, SAMP8, and a control counterpart strain, SAMR1. SAMP8 was originally derived from AKR/J strain, litters of which show the characteristic of cognitive decline with aging. These mice exhibit age-related deficits in learning and memory at an early age, and are considered a suitable animal model to study aging and memory deficit. Body weight, appearance, and plasma testosterone level of SAMR1 and SAMP8 at 12 weeks of age were determined. Body weight and appearance did not differ between SAMR1 and SAMP8, but plasma testosterone level in SAMP8 was lower than that in SAMR1 (Figure 1A). By determining the time required to find the platform (escape latency) as a function of days of training in the Morris water maze, we observed a marked decline in performance in SAMP8 compared with SAMR1 (Figure 1B). Because testosterone acts in part through aromatase-dependent conversion to estradiol, non-aromatizable dihydrotestosterone (DHT) was used to examine a direct role of androgens through androgen receptor (AR). SAMP8 treated with DHT showed significantly reduced escape latency time compared with untreated SAMP8. There was no difference in swim speed between the groups; however, % time in the quadrant was increased in DHT-treated SAMP8 (Figure 1B). These results indicate that DHT treatment ameliorated cognitive dysfunction in SAMP8. The water-maze is appropriate for hippocampal-dependent paradigms. However, DHT administration may affect behavior and how animals respond to different stimuli. Therefore, we performed an open field test to examine locomotion, exploratory behavior, and anxiety. No significant effect of DHT on locomotor performance was observed in SAMR1 and SAMP8, whereas SAMR1 moved significantly more compared with SAMP8 (Figure 1C). The ratio of the distance travelled in the central area to that in the total area in the open- field, an indirect measure of exploratory behavior and anxiety [15], was also observed. In SAMP8, DHT increased this ratio (Figure 1C), suggesting that DHT promoted exploratory behavior and diminished anxiety.

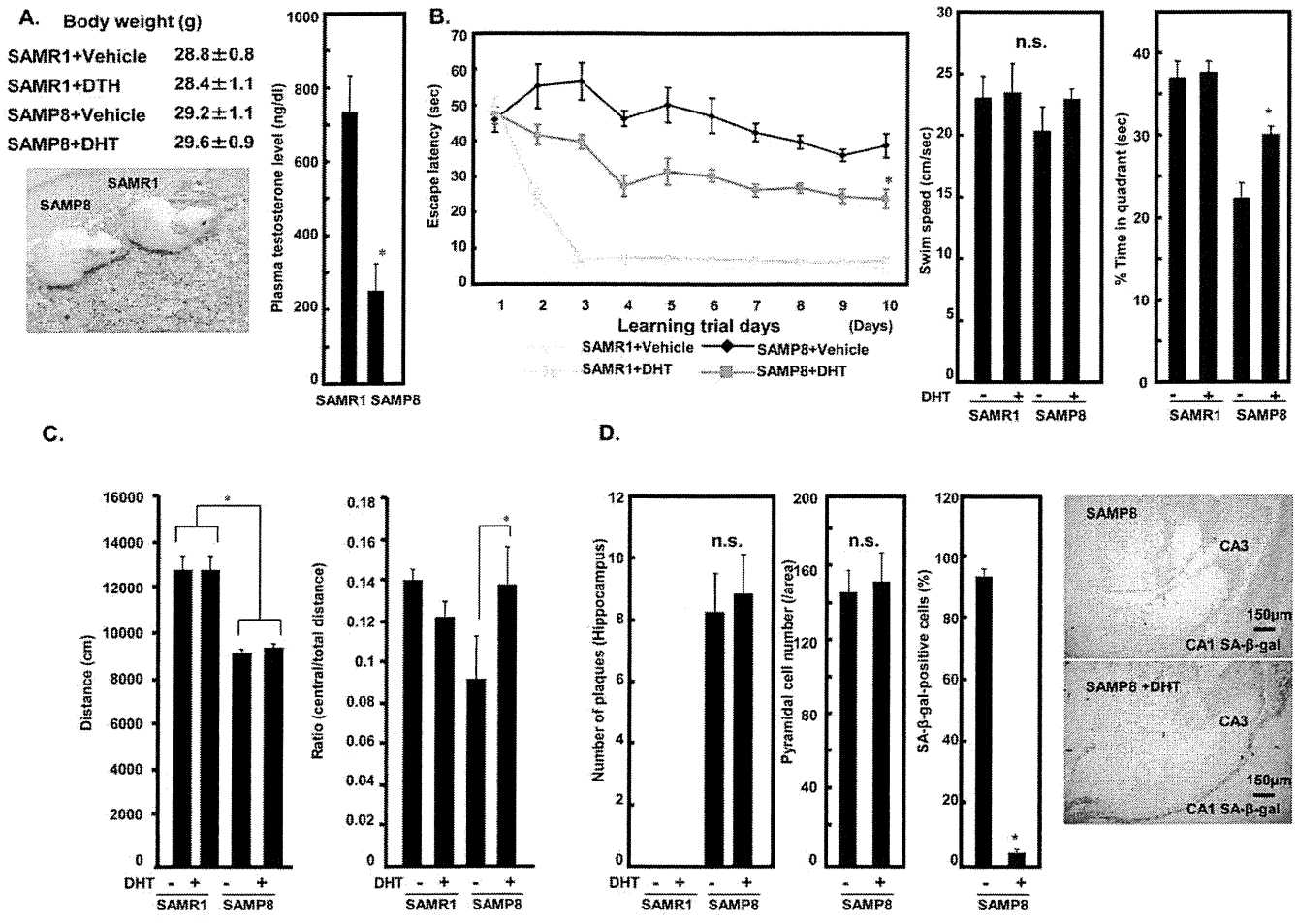

Figure 1. Testosterone deficiency causes senescence of hippocampus and cognitive impairment in SAMP8 mice. A. Body weight, appearance, and plasma testosterone level of male SAMR1 and SAMP8 mice at 12 weeks of age. **B.** Escape latency of SAMR1 (N = 10) and SAMP8 mice (N = 10). Male mice were treated daily for 2 weeks with DHT (500 μg s.c) before trials. Swim speed during quadrant test on day 10. **C.** Total distance and the ratio of central/total distance were measured in open field tests. **D.** Number of amyloid ß plaques, pyramidal cells, and SA-ßgal-positive cells in CA1 and CA3 areas of hippocampus in SAMR1 and SAMP8. (*p<0.05, n.s: not significant).
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Next, we assessed the number of amyloid ß plaques, pyramidal cells, and SA-ßgal-positive cells in CA1 and CA3 areas of the hippocampus in these mice (Figure 1D). The number of plaques was increased in SAMP8 compared with SAMR1, but was unaltered by treatment with DHT. The number of SA-ßgal-stained cells was significantly increased in SAMP8 compared with SAMR1, but treatment with DHT prevented this in SAMP8 despite no difference in pyramidal cell number (Figure 1D).

DHT treatment increased protein and mRNA expression of SIRT1 in SAMP8

Furthermore, to estimate the role of testosterone deficiency in SAMP8, we examined the effect of testosterone supplementation on cognitive function in much older SAMR1 and SAMP8. Similarly to young mice, we observed a marked decline in performance in SAMP8 compared with SAMR1 at 18 months of age. SAMP8 implanted with testosterone pellets showed significantly reduced escape latency time compared with placebo-treated SAMP8 (Figure 2A). Plasma testosterone level in SAMP8 at 18 months of age was lower than that in SAMR1, but implanted mice showed recovery to the level in young mice (Figure 2A). These results indicated that similar to DHT, testosterone also showed the improvement of cognitive function in SAMP8. Next, we examined the cause of low plasma testosterone in SAMP8. SAMP8 showed no testicular atrophy (Figure S1A), but more senescent phenotypes in Leydig cells, which produce testosterone in testes, than SAMR1 (Figure 2B). Moreover, we tried to allotransplant testes from SAMR1 to SAMP8 (Figure S1B). Although performance gradually responded to treatment up to 8–10 weeks, castrated SAMR1 showed a marked decline in performance whereas recipient SAMP8 showed cognitive improvement (Figure 2C).

As recently reported, overexpression or activation of SIRT1 inhibits cellular senescence and protects cellular function in various cell lines [13,16]. Therefore, we examined SIRT1 expression in the hippocampus of SAMP8 with or without DHT treatment, at 12 weeks of age. DHT treatment increased the protein and mRNA expression of SIRT1 in SAMP8 (Figure 2D). To investigate further the involvement of AR, we examined the expression of AR in SAMR1 and SAMP8 brains. The expression of AR was more abundant in the hippocampus than in other brain regions of SAMR1 and SAMP8 (Figure 2E).

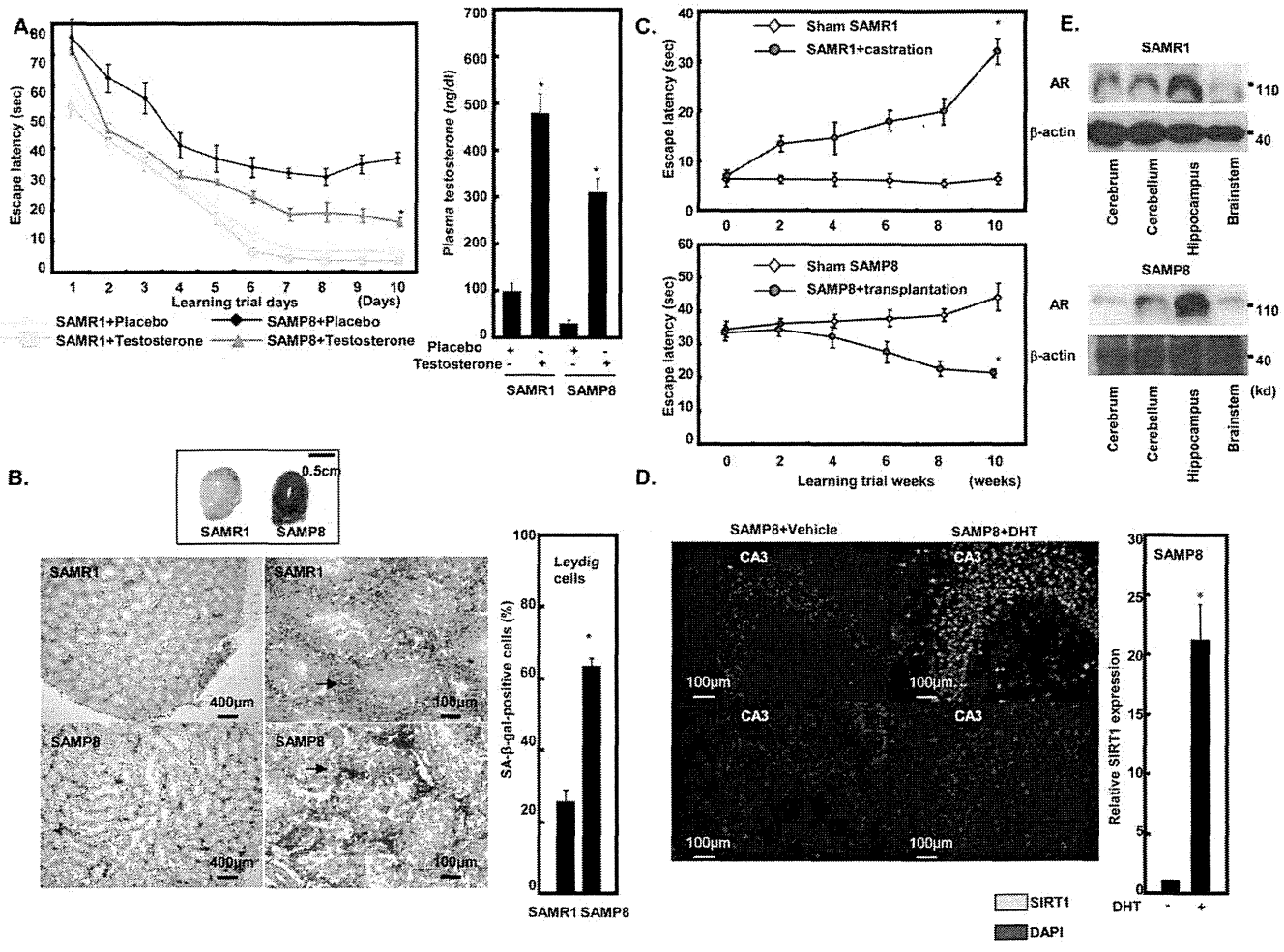

Figure 2. Supplementation of testosterone improves cognitive function in SAMP8 mice. A. Escape latency and plasma testosterone level of male SAMR1 (N = 10) and SAMP8 mice (N = 10) at 18 months of age. These mice were implanted subcutaneously with a placebo or a 21-day-release 2.5 mg testosterone pellet in the dorsal neck. **B.** Number of SA-βgal-stained Leydig cells in testes in SAMR1 and SAMP8. Arrows indicate Leydig cells. Representative SA-βgal-stained testes from SAMR1 and SAMP8. **C.** Escape latency of castrated SAMR1 (upper, N = 5) and recipient SAMP8 (lower, N = 5). Observation (0–10 weeks) was started from 3 weeks after operation. **D.** SIRT1 expression in hippocampus of SAMP8 with or without DHT treatment. Immunofluorescent staining for SIRT1 (green) and DAPI (blue). **E.** Expression of AR in SAMR1 and SAMP8 brains. (*p<0.05).
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Oxidative stress was increased in hippocampal cells of SAMP8

Oxidative stress may be closely related to senescence and age-related diseases. Also, an increase in oxidative stress has been suggested to be one of the earliest pathological changes in the brain in conditions with cognitive impairment such as AD [17]. Then, we examined the level of oxidative stress, using the SAMR1 and SAMP8 hippocampus at 12 weeks of age. SAMP8 hippocampus showed an increase in the level of oxidative stress compared with SAMR1 as judged by detection of carbonylated proteins. DHT treatment decreased carbonylated proteins in the SAMP8 hippocampus (Figure 3A). In parallel, the concentration of the neurotransmitter acetylcholine in hippocampal lysates was decreased in SAMP8 compared with that in SAMR1, and DHT treatment prevented this (Figure 3B).

Testosterone and DHT acts on vascular endothelial cells and stimulates the PI3K/Akt pathway, leading to eNOS activation through direct interaction of AR [18,19]. The eNOS/SIRT1 axis is recognized as one of the fundamental determinants of endothelial senescence, and SIRT1 acts as a driver of cellular stress resistance [20]. To examine the influence of DHT treatment on endothelial cells, we determined the degree of senescence and the expression of SIRT1 in endothelial cells around the CA3 area of the hippocampus. DHT-treated SAMP8 showed a reduction of SA-βgal-stained endothelial cells and increased SIRT1 expression compared to untreated SAMP8 (Figure 3C and D). To confirm that these cells were endothelial cells, not neuronal cells, cerebral microvessels were isolated from SAMR1 and SAMP8. In parallel with immunohistological staining, SAMP8 showed a reduction of SIRT1 expression compared to SAMR1, and DTH treatment increased SIRT1 expression compared to that in untreated SAMP8 (Figure 3E). These results suggest that vascular endothelial senescence in the hippocampus may be related to the memory deficit in SAMP8. Since testosterone and DHT activates eNOS, a NOS inhibitor, $\mathcal{N}^G$-nitro-L-arginine methyl ester hydrochloride (L-NAME), and N^5-(1-lmino-3-butenyl)-L-ornithine (L-VNIO), a

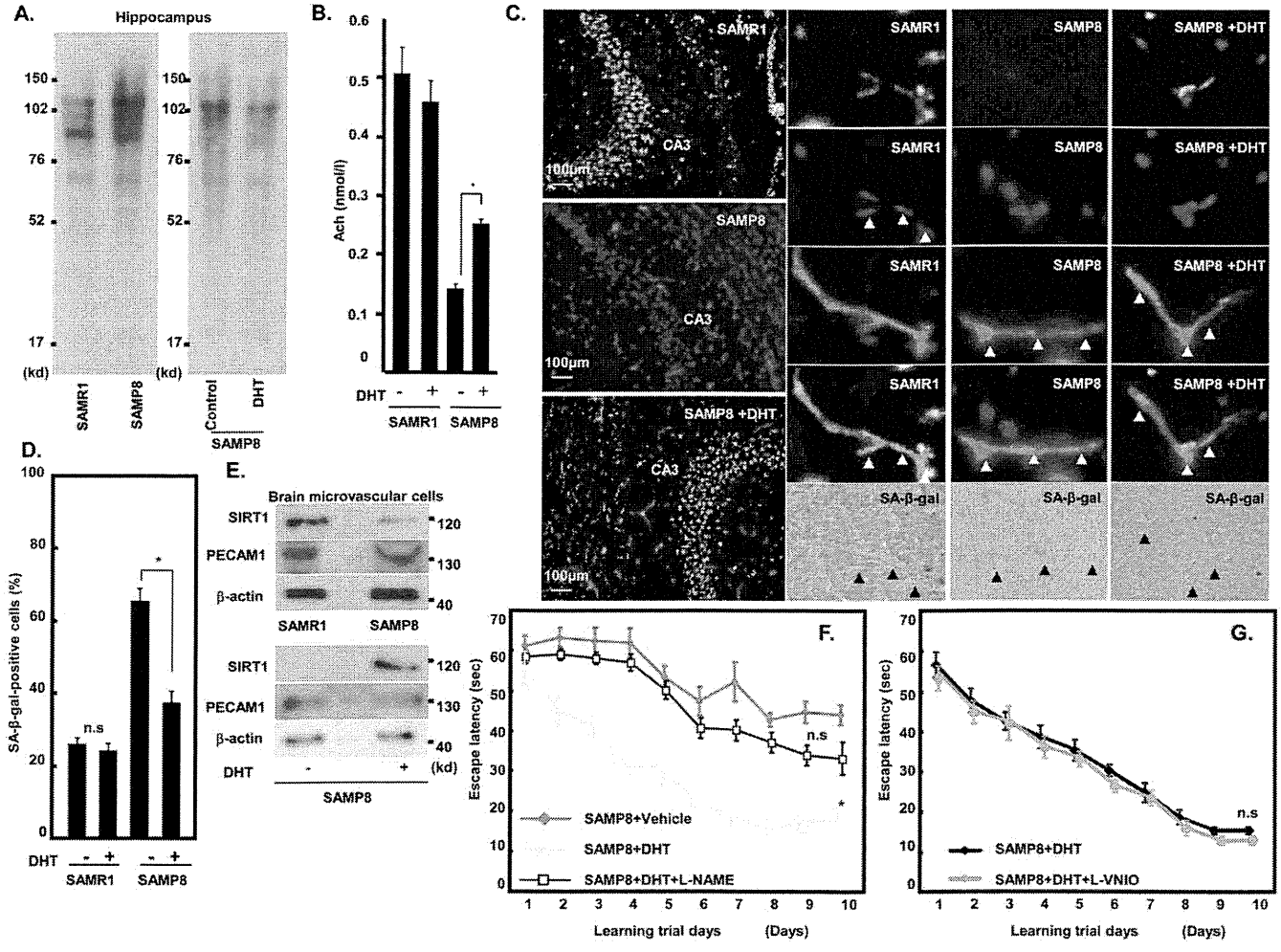

Figure 3. Senescent endothelial cells of hippocampus are decreased by treatment with DHT. A. Oxidative stress level was measured by detection of carbonyl groups introduced into proteins. **B.** Acetyl-choline concentration was measured by a colorimetric method. **C.** SA-βgal-stained endothelial cells and SIRT1 expression in CA3 area of hippocampus in SAMR1 and SAMP8 with or without DHT treatment. Immunofluorescent staining for SIRT1 (green), PECAM-1 (red), and DAPI (blue). **D.** Number of SA-βgal-stained endothelial cells in CA3 area of hippocampus in SAMR1 and SAMP8 with or without DHT treatment. **E.** Expression of SIRT1, PECAM-1, and β-actin was analyzed using cerebral micro vascular cells. **F.** Escape latency of SAMR1 (N = 10) and SAMP8 mice (N = 10). Male mice were treated daily for 2 weeks with DHT (500 μg s.c) and L-NAME (20 mg/kg gavage) before trials. **G.** Escape latency of SAMR1 (N = 5) and SAMP8 mice (N = 5). Male mice were treated daily for 2 weeks with DHT (500 μg s.c) and L-VNIO (5 mg/kg IP) before trials. (*$p<0.05$, n.s: not significant).
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selective neuronal NOS (nNOS) inhibitor, were applied to examine the involvement of NOS in this process. L-NAME abrogated the effects of DHT on cognitive function (Figure 3F). In contrast, L-VNIO did not change the effect of DHT (Figure 3G). These results suggest that eNOS/SIRT1 in endothelial cells may play an important role in the protective effect of testosterone against senescence of the hippocampus.

SIRT1 plays an important role in the protective effect of testosterone against endothelial senescence

Following the animal experiments, we examined whether testosterone inhibited endothelial senescence *in vitro* using cultured cells. We induced premature endothelial senescence by addition of H_2O_2 100 μmol/L for 1 hour. DHT or testosterone treatment inhibited SA-βgal activity and the morphological appearance of senescence (Figure 4A). We observed that oxidative stress decreased eNOS and SIRT1 and increased PAI-1 expression, and DHT or testosterone treatment prevented these changes and increased the phosphorylation of eNOS at Ser1177 (Figure 4B). Overexpression of SIRT1 significantly inhibited oxidative stress-induced senescence, and DHT accelerated the effect of SIRT1 through phosphorylation of eNOS at Ser1177 (Figure 4C). To determine the role of endogenous SIRT1, DHT-treated endothelial cells were transfected with SIRT1 siRNA or treated with sirtinol, a chemical inhibitor of SIRT1. SIRT1 siRNA or sirtinol abrogated the effect of DHT on SA-βgal activity (Figure 4D). We previously reported that testosterone activated eNOS [18], and eNOS activation promoted SIRT1 expression [21]. Accordingly, we examined the role of eNOS in the protective effect of testosterone. We observed that DHT or testosterone treatment increased NOS activity that was reduced by oxidative stress (Figure 4E). Treatment with eNOS siRNA or L-NAME decreased the inhibitory effect of DHT on a senescent phenotype in parallel with SIRT1 expression (Figure 4F and G). These results indicate that eNOS/SIRT1 play an important role in the protective effect of testosterone and DHT against a senescent phenotype.

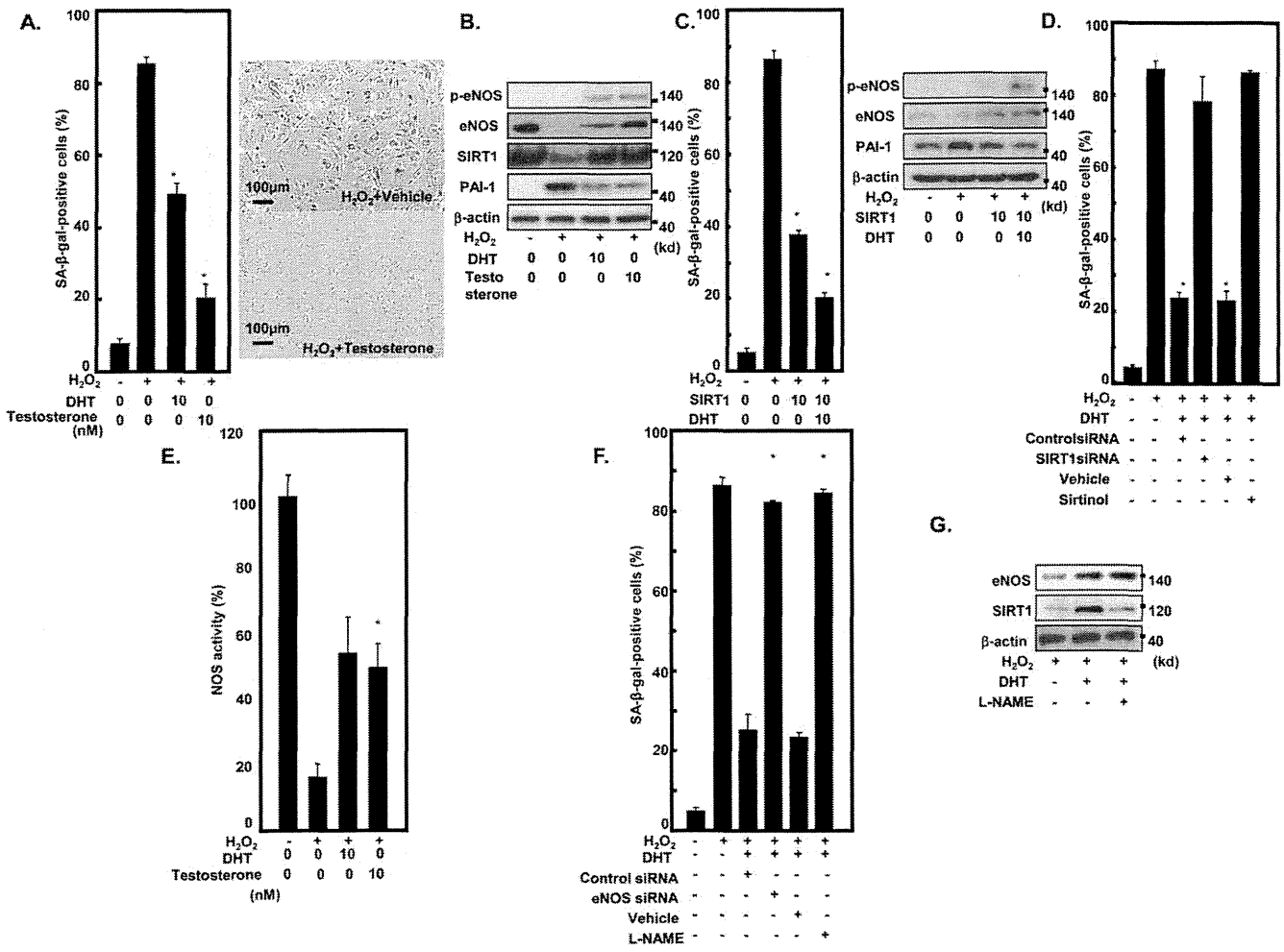

Figure 4. Testosterone inhibits oxidative stress-induced endothelial senescence through eNOS/SIRT1. A. Testosterone inhibited SA-βgal activity and senescent morphological appearance induced by hydrogen peroxide (100 μmol/L). **B.** Expression of eNOS, SIRT1, and PAI-1 in hydrogen peroxide (100 μmol/L)-treated HUVEC under treatment with DHT or testosterone. **C.** Overexpression of SIRT1 and DHT reduced SA-βgal activity. eNOS expression was increased by overexpression of SIRT1, and DHT increased phosphorylation of eNOS (Ser1177). **D.** SIRT1 inhibition by siRNA or sirtinol (100 μmol/L) abrogated the effect of testosterone on SA-βgal activity. **E.** Treatment with testosterone or DHT increased eNOS activity. **F.** eNOS inhibition by siRNA or L-NAME (10 mM) abrogated the effect of testosterone on SA-βgal activity. **G.** Treatment with L-NAME decreased SIRT1 expression in DHT-treated HUVEC. (*$p<0.05$, $N=3$).
doi:10.1371/journal.pone.0029598.g004

Senescent endothelial cells induced by oxidative stress promoted neuronal senescence

Finally, we hypothesized that endothelial senescence promotes senescence of adjacent neuronal cells. To test this hypothesis, we used a co-culture system of endothelial cells (HUVEC) with neuronal cells (mouse hippocampal neuronal cells; MHC) (Figure 5A). Both cells were co-cultured, but were separated by a microporous polycarbonate membrane, for 10 days after endothelial cells were treated with hydrogen peroxide, and the senescent phenotype of MHC was analyzed. We found that the number of SA-βgal-positive cells and the senescent appearance of MHC were increased, and the concentration of acetylcholine in cells was decreased by co-culture with senescent endothelial cells (Figure 5B). In parallel with this, MHC showed increased PAI-1 and p53, and decreased SIRT1 expression (Figure 5C). We also found that senescent endothelial cells showed increased expression of inflammatory cytokines such as IL-6, IL-8, MCP-1, and TNF-α (Figure 5D). Both MHC and HUVEC, or HUVEC alone were treated with testosterone at 3 days before HUVEC were treated with hydrogen peroxide, and both cells were co-cultured for 10 days, and the senescent phenotype of MHC was analyzed. We found that the number of SA-βgal-positive MHC was decreased by treatment of HUVEC with testosterone irrespective of the treatment of MHC with testosterone (Figure 5E). In addition, we found that a SIRT1 activator, resveratrol treatment rescued the senescent phenotype of MHC (Figure 5F). These results suggest that senescent endothelial cells exhibit a senescence-associated secretory phenotype [22], induce neuronal senescence, and testosterone rescues it through up-regulation of SIRT1 (Figure 5G).

Discussion

Testosterone level and cognitive function show a decline with age in men. A series of evidence suggests that this association is not just age related [23]. Results from cell culture and animal studies provide evidence that testosterone could have protective effects on brain function, especially in the hippocampus [24]. Here, we demonstrated that administration of testosterone restored cognitive function in male SAMP8 in association with improvement of the senescent phenotype in the hippocampus and cerebral vessels.

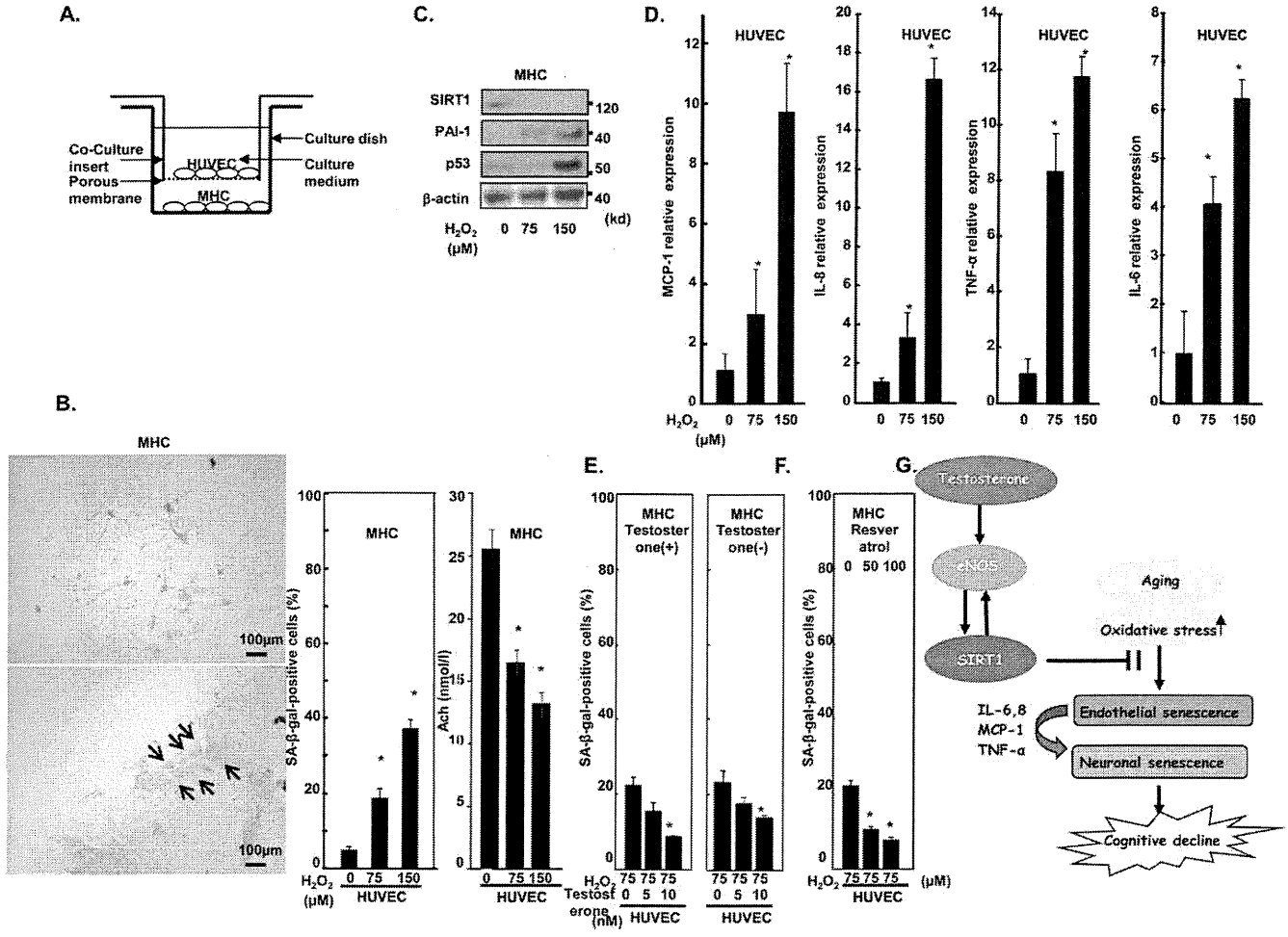

Figure 5. Oxidative stressed-induced endothelial cell senescence promotes adjacent neuronal cell senescence. A. Co-culture cell culture dish. **B.** Number of SA-βgal-stained MHC and senescent appearance of MHC were increased, and acetyl-choline concentration was decreased by co-culture with senescent endothelial cells. Senescent MHC are indicated by arrows. **C.** Expression of SIRT1, PAI-1, p53, and β-actin in MHC co-cultured with senescent endothelial cells. **D.** Expression of IL-6, IL-8, MCP-1, and TNF-α in endothelial cells were analyzed by RT-PCR. **E.** The number of SA-βgal-stained MHC was decreased by treatment with testosterone in both MHC and HUVEC (MHC, testosterone (+)), or HUVEC (MHC, testosterone (−)) alone. **F.** Resveratrol decreased the number of SA-βgal-stained MHC co-cultured with senescent endothelial cells. (*$p<0.05$, N=3). **G.** Hypothetical signal transduction pathways of testosterone in endothelial cells.
doi:10.1371/journal.pone.0029598.g005

We also showed that testosterone ameliorated endothelial senescence through eNOS/SIRT1-dependent mechanisms *in vitro*. The present study demonstrated that testosterone and SIRT1 interacts with each other and inhibited the senescence of hippocampal vascular and neuronal cells, suggesting that testosterone replacement therapy is a treatment option for cognitive decline with aging.

Testosterone may act in part through aromatase-dependent conversion to estradiol. To estimate a direct effect of androgens through AR, testosterone and DHT were used in this study. Both compounds showed significant protective effects on cognitive function.

In the present study, we used SAMP8 mice. SAMP is comprised of 14 strains derived from selective inbreeding of the AKR/J strain. SAMP8 exhibits age-related learning and memory deficits, as well as amyloid-like deposits in the brain [25]. Increased expression of hyperphosphorylated tau has also been detected in SAMP8 [26]. Given such features, SAMP8 has been proposed as a plausible age-associated AD animal model, and a suitable rodent model for studying the molecular mechanism underlying cognitive impairment [27]. A previous study has shown an age-related decrease in serum testosterone in SAMP8, and suggesting that impaired cognitive function in SAMP8 is due to reduced testosterone [28]. We observed that AR expression was abundant in the hippocampus of SAMR1 and SAMP8. Several studies have demonstrated that testosterone has a neuroprotective effect through AR in the hippocampus [29,30], and testosterone induced NO productions via AR-dependent activation of eNOS in endothelial cells [18,19].

Accumulating evidence suggests that NAD^+-dependent deacetylase SIRT1 play an essential role for cellular senescence and cognitive function. SIRT1 modulates endothelial cellular senescence [13], and overexpression of SIRT1 exhibits neuroprotective effects in hippocampus, and cognitive function of *Sirt1*-KO mice is markedly impaired [10,31,32].

The precise etiologic mechanism of the cognitive decline with aging is unclear, but it has been identified that cardiovascular risk factors are associated with a higher incidence of cognitive impairment [33]. In addition, age-associated vascular inflammation is an early manifestation of chronic stress responses, i.e. overloading of ROS on endothelial cells [34]. Indeed, SAMP8 showed enhancement of oxidative stress and a senescent phenotype in the hippocampus. Notably, senescent endothelial cells were increased in the hippocampus of SAMP8 accompanied by a reduction of SIRT1, and L-NAME abrogated the effect of DHT on cognitive function. Therefore, we hypothesized that testosterone influenced cerebral endothelial senescence via eNOS/SIRT1, and that pro-inflammatory cytokines, which were derived from senescent endothelial cells, promoted senescence in adjacent neuronal cells. Indeed, we observed that testosterone induced eNOS activity, and subsequently increased SIRT1 expression in endothelial cells. Inhibition of eNOS/SIRT1 abrogated the effect of testosterone on endothelial senescence. In a co-culture system, we found that senescent endothelial cells promoted senescence of adjacent neuronal cells, and treatment of endothelial cells with testosterone inhibited senescence of adjacent neuronal cells. It can reasonably be speculated, therefore, that SIRT1 may exert salutary actions against cognitive decline with aging by preventing a senescence-associated secretory phenotype of endothelial cells. Because L-NAME is a non-selective inhibitor of NOS, it is possible that the effect of L-NAME might be in part a result of inhibition of nNOS in concert with eNOS. However, a specific nNOS inhibitor, L-VNIO did not change the effect of DHT in SAMP8. In co-culture experiments, we found that treatment with resveratrol or testosterone did not change the expression or activation of nNOS in MHC (Figure S1C and D). Further studies are needed to address the differential role of eNOS and nNOS, and the exact role of SIRT1 *in vivo*.

In conclusion, supplementation of testosterone prevented cognitive impairment of SAMP8, in which testosterone secretion was decreased in association with the senescence of testis Leydig cells, through an eNOS/SIRT1-dependent mechanism. Unprecedented reversal of the senescent hippocampal changes and vascular protection may justify exploration of a neuronal rejuvenation strategy by utilizing testosterone for the prevention of cognitive decline with aging, particularly through up-regulation of eNOS/SIRT1.

Methods

Materials

Dihydrotestosterone (DHT), testosterone, and N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME) were purchased from Sigma (St. Louis, MO). Hydrogen peroxide (H_2O_2) and resveratrol were purchased from Wako Pure Chemical Industries (Osaka, Japan). Testosterone and placebo pellets were purchased from Innovative Research of America (Sarasota, FL). N^5-(1-Imino-3-butenyl)-L-ornithine (L-VNIO) was purchased from Enzo Life Sciences (Plymouth Meeting, PA).

Cell culture

Human umbilical vein endothelial cells (HUVEC) were purchased from CAMBREX (Walkersville, MD). Population doubling levels (PDL) were calculated as described previously [35], and all experiments were performed at PDL of 10–11. In our preliminary experiments, HUVEC were cultured in EBM without phenol red (Clonetics, Walkersville,MD) with 10% dextran-charcoal-stripped serum to remove steroids from the culture medium. This condition, however, induced marked growth arrest and an increase in senescent cells. Consequently, we performed all experiments in EBM-2 (Clonetics) with 10% complete serum-supplemented medium.

Animal experiments

The animal experiments were approved by our institutional review board (animal experiments ethics board, Graduate School of Medicine and Faculty of medicine, The university of Tokyo (approval ID: M-P-09-056)). Senescence-accelerated mice prone (SAMP) 8 and control senescence-accelerated mice resistant (SAMR) 1 male mice were all housed and maintained in a room at 22±2°C with automatic light cycles (12 h light/dark) and relative humidity of 40–60%. Mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). Food and tap water were provided ad libitum throughout the study. In the water maze test of this study, a group of male SAMR1 (N = 10) and SAMP8 (N = 10) was first tested. Male mice of 12 weeks of age were treated daily for 2 weeks with DHT (500 μg in 0.05 ml/mouse) by subcutaneous injection (s.c.) in the neck before the water maze test. Male mice of 18 months of age underwent subcutaneously implantation of a placebo (N = 5) or a 21-day-release 2.5 mg testosterone (N = 5) pellet into the dorsal neck region. L-NAME was given by gavage once a day (20 mg/kg) [36]. L-VNIO was given by intraperitoneal injection (0.5 mg/kg) [37]. Small fragments of testis tissue fragments from SAMR1 were grafted under the back skin of castrated male SAMP8 as previously described [38]. Briefly, after removal of the capsule and obvious connective tissue, donor testes were cut into small fragments. Testis fragments were kept in Dulbecco's modified Eagle's medium

(Gibco Lab Inc., Grand Island, NY, USA) on ice until grafting. SAMR1 were anesthetized and castrated, and testicular tissue fragments were grafted under the back skin of SAMP8. Mice were anesthetized with enflurane, killed by cervical dislocation, and trunk blood collected within 1 min. The blood was centrifuged and plasma testosterone was measured by radioimmunoassay method. The brain was removed for histological examination, after systemic perfusion with phosphate-buffered saline (PBS). For immunohistochemical studies, mouse brains were processed and labeled with anti-amyloid-β antibody (Immuno-Biological Laboratories Co., Ltd., Gunma, Japan) to visualize extracellular amyloid plaques, anti-NeuN antibody (Millipore, Billerica, MA) to assess pyramidal cell number, or DAPI (Dojindo Molecular Technologies, Inc., Tokyo, Japan) for nuclear staining. The primary antibody was purified rat anti-mouse CD31 (platelet endothelial cell adhesion molecule; PECAM-1) monoclonal antibody from Pharmingen (San Jose, CA, USA). Secondary antibodies (Alexa Fluor 488 donkey anti-rat IgG and Alexa Fluor 594 donkey anti-rat IgG) and antifade reagent were from Molecular Probes (Invitrogen). Fluorescent images were analyzed using a fluorescence microscope (BZ-9000, KEYENCE, Osaka, Japan).

Plasmids and siRNA transfection

Proliferating cells were washed three times with growth medium and exposed to the indicated concentrations of testosterone or DHT diluted in medium. pIRES-SIRT1 plasmid was provided by Dr. M. Takata [39], and Dr. R.A. Weinberg [40]. Each plasmid was overexpressed by transfection using Lipofectamine LTX and PLUS reagents (Invitrogen) for HUVEC according to the manufacturer's instructions. Proliferating cells were transfected with each siRNA using silMPORTER (Upstate Cell Signaling Solutions). siRNAs for SIRT1 (GAT GAA GTT GAC CTC CTC A [41] and TGA AGT GCC TCA GAT ATT A), and eNOS were purchased from Santa Cruz Biotechnology, Inc.

Immunoblotting and immunoprecipitation

Cells were lysed on ice for 1 hour in buffer (50 mmol/L Tris-HCl, pH 7.6, 150 mmol/L NaCl, 1% NP-40, 0.1% SDS, 1 mmol/L dithiothreitol, 1 mmol/L sodium vanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin and 10 mmol/L sodium fluoride). Equal amounts of protein were separated by SDS/PAGE gel electrophoresis and transferred to nitrocellulose membranes. After blocking, the filters were incubated with the following antibodies; anti-SIRT1, anti-nNOS, anti-AR (Cell Signaling, Danvers, MA), anti-eNOS (BD Transduction Laboratories, San Jose, CA), anti-PAI-1 (Molecular Innovations, Southfield, MI), anti-PECAM-1 (Santa-Cruz Biotechnology, CA), and anti-ß-actin (Sigma). After washing and incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Amersham, Piscataway, NJ) for 1 hour, antigen-antibody complexes were visualized by using an enhanced chemiluminescence system (Amersham).

Senescence-associated ß-galactosidase (SA-ßgal) staining

HUVEC were pretreated with diluted EGM-2 medium for 3 day. HUVEC were then washed three times with EGM-2 and treated for 1 hour with 100 μmol/l H_2O_2 diluted in EGM-2. After treatment, HUVEC were trypsinized, re-seeded at a density of 1×10^5 in 60-mm dishes, and cultured with EGM-2 containing DHT or testosterone for 10 days. The proportion of SA-ßgal-positive cells was determined as described by Dimri et al [42].

NOS activity assay

NOS activity was determined using an NOS assay kit (Calbiochem) according to the manufacturer's instructions.

Measurement of acetylcholine

The concentration of acetylcholine was measured with a choline/acetylcholine quantification kit (BioVision, CA, USA) according to the manufacturer's instructions.

Real-time quantitative reverse transcription PCR

Total RNA was isolated with ISOGEN (Nippon Gene Inc., Toyama, Japan). After treatment with Rnase-free Dnase for 30 min, total RNA (50 ng/μl) was reverse transcribed with random hexamers and oligo d(T) primers. The expression levels of SIRT1, IL-6, IL-8, MCP-1, and TNF-α relative to ß-actin were determined by means of staining with SYBR green dye and a LineGene fluorescent quantitative detection system (Bioflux Co., Tokyo, Japan). The following primers were used: SIRT1 F 5′-CCTGACTTCAGGTCAAGGGATGGTA-3′, R 5′-CTGATTAAAAATATCTCCTCGTACAG-3′; ß-actin F 5′-TGGGCATGGGTCAGAAGGAT-3′, R 5′-AAGCATTTGCGGTGGACCAT-3′; IL-6 F 5′-GGGAAGGTGAAGGTCGG-3′, R 5′-TGGACTCCACGACGTACTCAG-3′, IL-8 F 5′-CTGGCCGTGGCTCTCTTG-3′, R 5′-CCTTGGCAAAACTGCACCTTT-3′; TNF-α F 5′-GTAGCCCACGTCGTAGCAAAC-3′, R 5′-CTGGCACCACTAGTTGGTTGTC-3′; MCP-1 F 5′-CATTGTGGCCAAGGAGATCTG-3′, R 5′-CTTCGGAGTTTGGGTTTGCTT-3′.

Co-culture system

For these experiments, co-culture dishes were used as outlined in Figure 5A. They were obtained from BD Biosciences (Erembodegem, Belgium) with a 6-well format. HUVEC were treated with H_2O_2 (100 μM) for 1 h and cultured on the permeable microporous (0.4 μm) membrane in the insert, and mouse hippocampus neuronal cells on the base of the culture dish, kept physically separated but allowing the passage of micromolecules through the porous membrane for 10 days. Mouse hippocampus neuronal cells were purchased from DS Pharma Biomedical Inc. (Osaka, Japan).

Quantitative analysis of amyloid β

Measurement of amyloid β was performed using an amyloid β (1–40) (FL) assay kit (Immuno-Biological Laboratories Co., Ltd., Gunma, Japan) according to the manufacturer's instructions.

Morris water maze test

The procedure of the Morris water maze test was described previously [43]. SAMR1 and SAMP8 mice were trained to find a visible platform with three trials on the first day, and then tested to find the hidden platform for 10 consecutive days. In each trial, the mice were allowed to swim until they found the hidden platform, or until 2 min had passed, and the mouse was then guided to the platform. On the test days, the platform was hidden 1 cm beneath the water. The escape latency was recorded by a video camera. The swim speed of each mouse was calculated by means of a video tracking system. Probe tests were performed on the 10^{th} day. During percent time quadrant test, the platform was removed from the pool. Mice were started in a position opposite the location of the platform position and allowed to swim for 60 seconds.

Open field test

The open field test fear response to novel stimuli was used to assess locomotion, exploratory behavior, and anxiety. Open field test protocols were modified from that of Lukacs et al [44]. The open field test consisted of a wooden box (60×60×60 cm) and was indirectly illuminated by two fluorescent lights. A 10 cm area near the surrounding wall was delimitated and considered the periphery. The rest of the open field was considered the central area. The distance travelled, the ratio of the distance travelled in the central area/total distance travelled, and the time in the center of the open- field were analyzed as a measure of anxiety-like behavior. During the test, mice were allowed to move freely around the open field and to explore the environment for 15 min.

Isolation of cerebral microvessels

Cerebral microvessels were isolated from the remaining brain tissue as previously described by Zhang et al [45] with minor modifications. Brain tissue, devoid of large vessels, was homogenized in ice cold PBS with Dounce homogenizer and centrifuged twice at 2000 g at 4°C. The supernatant, containing the parenchymal tissue, was discarded. The pellet was resuspended in PBS and centrifuged as described above. The resulting pellet was resuspended and layered over 15% Dextran (in PBS) (Sigma, St.Louis, MO) and centrifuged at 4500 g for 30 minutes at 4°C. The top layer was aspirated and discarded and the remaining pellet resuspended in 15% Dextran and centrifuged. The final pellet was resuspended in 1% bovine serum albumin (BSA), the suspension was then passed though a 40-µm nylon mesh (BD Falcon). Microvessels retained on the mesh were washed with BSA/PBS and collected by centrifugation at 900 g for 10 minutes at 4°C.

Data analysis

Values are shown as mean ± S.E.M in the text and figures. Differences between the groups were analyzed using one-way analysis of variance, followed by Bonferroni test. Probability values less than 0.05 were considered significant.

Supporting Information

Figure S1 Testes of SAMP8 and SAMR1 mice and role of nNOS in neuronal senescence. A. Testis weight of SAMR1 and SAMP8 with or without testosterone. **B.** Photographs of SAMR1 donor and SAMP8 recipient mice. White arrows indicate operation scar. **C.** Expression of nNOS in MHC treated with resveratrol or testosterone under the oxidative stress. **D.** Activity of nNOS in MHC treated with resveratrol or testosterone under the oxidative stress. ($^{*}p<0.05$, N = 3, n.s: not significant).
(TIF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: HO MA YO. Performed the experiments: HO TA. Analyzed the data: HO SO KI ME MA. Contributed reagents/materials/analysis tools: TK MS. Wrote the paper: HO MA.

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速報論文

Short Paper

高齢者におけるウェアラブル血圧センサーの臨床応用：～認知機能およびストレス感受性からみた血圧短期変動評価への有用性の検討～

Validity and Usefulness of 'Wearable Blood Pressure Sensing' for Detection of Inappropriate Short-Term Blood Pressure Variability in the Elderly: Impact of Cognitive Function and Stress Response

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Summary

An increase in short-term blood pressure (BP) variability is a characteristic feature in the elderly. It makes the management of hemodynamics more difficult, because it is frequently seen disturbed baro-reflex function and increased arterial stiffness, leading to isolated systolic hypertension. Large BP variability aggravates hypertensive target organ damage and is an independent risk factor for the cardiovascular (CV) events in elderly hypertensive patients. Therefore, appropriate control in BP is indispensable to manage lifestyle-related diseases and to prevent subsequent CV events. In addition, accumulating recent reports show that excessive BP variability is also associated with a decline in cognitive function and fall in the elderly. In the clinical settings, we usually evaluate their health condition, mainly with single point BP measurement using cuff inflation. However, unfortunately we are not able to find the close changes in BP by the

traditional way. Here, we can show our advantageous approach of continuous BP monitoring using newly developing device 'wearable BP sensing' without a cuff stress in the elderly. The new device could reflect systolic BP and its detailed changes, in consistent with cuff-based BP measurement. Our new challenge suggests new possibility of its clinical application with high accuracy.

1. はじめに

未曾有の高齢化が進む中で，高齢者の慢性疾患管理が重要になっている．なかでも高血圧罹患率は非常に高く，高血圧を基盤とする様々な疾病を予防するには普段からの厳格な管理が必要となる．その厳格な管理を達成するためには，まず高齢者高血圧の特徴を熟知する必要がある．高齢者は大動脈から中小の筋性動脈を中心に「動脈壁硬化」を呈する．いわゆる血管老化と考えられる現象である．その背景には石灰沈着，過剰なコラーゲン沈着，そして弾性線維の主成分であるエラスチンの脱落・変性・断片化など，様々な変化が起こっている．この動脈壁硬化がより進むことによりWindkessel(ふいご)機能が低下し，孤立性収縮期高血圧を呈しやすくなってくる[Iijima 09]．その結果，拡張期血圧はあまり高値を示さず，ある症例では冠動脈還流圧の低下が惹起される．また，もう一つの特徴として，圧受容器反射機能の低下などにより血圧の自動調節が破綻し，著明な血圧変動を起こしやすくなってくる．その過度の血圧変動が脳心血管疾患の発症や相対的臓器虚血を惹起し，特に相対的脳虚血の場合には立ちくらみやめまい，ひいては転倒リスクにまでつながる．これらの現象は高齢者本人の生活の幅や質(いわゆる日常生活活動度)を大きく損ねるきっかけにもなり得る．よって，「いかに高齢者の血圧変動をより詳細に(連続的に)，かつ簡便に評価し，そして臨床診療における高齢者の健康管理に活用するのか」ということが今後の大きな課題となる．

さて，本論文では，大量かつ連続のヘルスケアデータ(血圧データ)を扱う．カフ血圧が，現在の絶対的な医療基準であるため，これまで血圧変動は離散的にしか扱われてこなかったものである．今までにない情報なので，まずはデータをとってみることが重要である．大量に蓄積した血圧の結果を分析すれば，アラートを出すべき状態変化の議論につながるであろう．血圧の超短期変動のように，意味のある情報については，機械学習により自動検出ができるようアルゴリズムを組んで，実際のヘルスケアサービスにつながることを期待している．よって，連続的に血圧変動をモニタリングできるセンサーを用いて，実際の患者から連続データを大量に取得し，重要な血圧変動を描出することができたことを報告する．

2. 高齢者の高血圧と血圧変動

高齢者の高血圧における様々な特徴を表1に列挙する．まず加齢に伴い血管壁硬化が進み，Windkessel 機能が低下することにより，脈圧増大を伴う収縮期高血圧を呈しやすくなる．また，塩分摂取量や降圧薬の服薬管理状況にも大きく影響を受けやすい．さらに，高齢者の血圧管理を行う上で，単なる血圧値だけではなく，数多くの計測によるその血圧変動を十分考慮に入れた管理をしなければならない．いわゆる血圧変動には，交感神経活性や環境因子など様々な要因が考えられるが，なかでも24時間自由行動下自動血圧測定(ambulatory blood pressure monitoring: ABPM)から判定される「日内変動」と，本人が自宅において自動血圧計にて連日測定する「日間変動」の重要性が注目されている．

健常者は夜間就寝中においては(昼間に比べて)生理的に約 10～20%の降圧を示す．高齢者ではその生理的な夜間降圧のパターンが破綻しやすく，Non-dipper 型や Riser 型を呈する症例が少なくない．これらは脳・心血管系疾患のハイリスク群と位置づけされている．また，逆に夜間の過度降圧(Extreme-dipper)を呈する症例も数多く，通常の外来診療における単回の血圧測定による病態把握に限界が生じてくる．これらの日内変動や日間変動が大きいほど，脳・心血管系疾患発症のリスクが高いことは数多くの臨床研究によって報告されている[Kikuya 08]．

また，高齢者における短時間内の血圧変動として，起立性低血圧や食後低血圧が代表的である．図1に ABPM により高齢者高血圧の特徴を捕えられた症例を示す．夜間の血圧は相対的に Extreme-dipper 型を呈し，起床前後の時間帯は典型的な Morning surge を示している．そして，食後に急激な血圧低下も起こっていることが ABPM によって描出されている．

この結果からも，高齢者の血圧はかなり短時間の中でも劇的な変動を示しており，ある症例ではこの過度な血圧変動が相対的脳虚血を惹起し，いわゆるめまい・立ちくらみなどの症状を訴えやすくなり，最終的に易転倒性につながる．

表1 高齢者高血圧の特徴

1. 血管壁硬化(Windkessel(ふいご)機能の低下)
収縮期高血圧，脈圧の増大(→冠動脈還流圧の低下)
2. 血圧の動揺性
白衣高血圧，仮面高血圧
起立性低血圧，食後低血圧
(圧受容器時反射の低下，自律神経機能低下と関連)
3. 血圧変動の増加(日内変動・日間変動)
早朝高血圧(Morning surge)や生理的夜間血圧の破綻
・夜間非降圧型(Non-dipper)/夜間上昇型(Riser)
・夜間の過度降圧(Extreme-dipper)
4. 降圧や血圧変動に伴う臓器血流の低下(脳，心，腎臓)
5. 食塩感受性(体液量依存性)が高い
6. 服薬状況の安定性が低い(コンプライアンス不良)

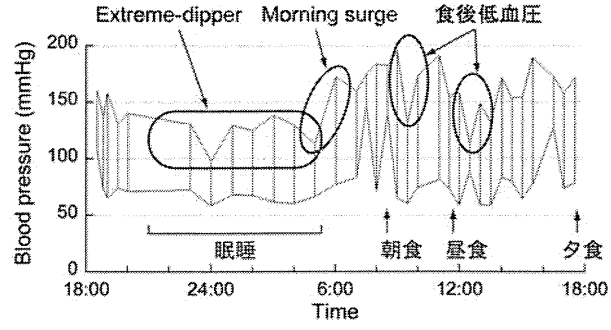

図1 ある高齢者の24時間自由行動下血圧(ABPM)の推移

さらに，高齢者では高血圧と認知機能との関連も無視できない．近年の報告では，認知症の発症・進展には高血圧も含めた生活習慣病との関連も注目されていることから，より幅広い病態把握が必要である[Sakakura 07]．高血圧の管理不良や過度の血圧変動は，急性の脳血管障害だけでなく，慢性の脳虚血所見（ラクナ梗塞や白質病変）も大きく増大させることから，認知症予防という観点からも高血圧管理，ひいては血圧変動の管理が必要になってくることは間違いない．それらを予防するためには，「個人差の大きい高齢者の血圧管理において，短時間内に起こる過度な血圧変動をいかに簡易に評価できるか」が大きな鍵となってくる．

3. ウェアラブル血圧センサーの高齢者への応用

従来のカフ式血圧測定では頻回な測定にも限界があり，同時にカフ圧迫という患者への負担も増える．実際の臨床現場では，医療機関における外来受診時の単回測定によって評価されており，個々の血圧変動の状態を把握することができていない現実がある．また，一般的にカフ式血圧測定が始まると被験者は行動を中断して安静にしなければならないという測定上の制限も出てくる．

東京大学大学院工学系研究科・山田一郎研究室はカフを必要とせずに脈波伝播速度を用いて連続的に血圧をモニタできる血圧計を開発し，臨床への応用を進めているところである[Labat 11]．この原理は脈波伝播速度を元に血圧を推定する方法を採用している[Lopez 10]．脈波伝播速度法では，心電の R 波と脈波の立ち上がり点の時間差である脈波伝達時間(Pulse Arrival Time: PAT)から，収縮期血圧値を算出することで血圧推定を行っている．脈波の計測部位としては，体動による変化を最低限に抑えられる目的で耳たぶを選択している．上記の基本計測原理の検証のため，エルゴメータを用いた自転車こぎ運動による評価実験に行い，医師によるカフ式手動血圧計での聴診法による測定結果と比較して，大きな乖離のない結果が得られている．

今回，我々は開発中であるカフレスで連続的に収縮期血圧を測定できるウェアラブル血圧センサーを用いて，高齢者での短期変動に注目し臨床実験を行った．特に，様々な負荷に対する短時間での昇圧変化，および起立などの急な降圧など，『超』短期変動に焦点を合わせウェアラブル血圧センサーの有用性を検討した．なお，ウェアラブルセンサーの詳細については，既報の論文[Lopez 10, Labat 11]を参照していただくこととして，本稿では詳細を省略する．

4. 実験方法

具体的には，高齢者の『超』短期変動を評価する目的で，表 2 に示すような様々な負荷（メンタル，歩行，立位など）を行い従来のカフ式血圧計（血圧モニタリング）も並行して測定し，ウェアラブル血圧センサーによるデータと比較した．カフ測定間隔に関しては高齢の対象者へのカフ圧迫による負担を軽減させることに配慮しながら，3～5 分間隔で測定した．装置は，通常 1 時間ごとに測定されるカフ式の自動血圧計（NIHON KOHDEN ベッドサイドモニタ BSM2301，OMRON デジタル自動血圧計 HEM-711 ファジィ，またはフクダ電子 FM-800）を用いたが，測定は手動で行った．

対象は東京大学附属病院・老年病科に生活習慣病の慢性管理目的もしくは物忘れを主訴の一つとして入院された 60 歳以上の症例とし，悪性腫瘍や急性疾患を持ち合わせる症例は除外した．すべての症例に同意書を取得した．

5. 結果および考察

5.1 メンタル・ストレス反応性の血圧変動の結果

この『超』短期変動に焦点を合わせ，現在までに 50 症例（平均年齢 80 歳±5.8 歳：男性 16 例，女性 34 例）に対してデータ収集を行った．メンタル・ストレス負荷の中で「2つの物語に対する暗記（復唱）」負荷に対してウェアラブル血圧センサーにより昇圧が認められた症例は 50 例中 20 例（40%），「計算（暗算）」負荷による昇圧を示した者は 50 例中 24 例（48%）であった．また，歩行負荷による昇圧は 50 例中 33 例（66%）であり，この血圧センサーにより比較的多くの症例に超短期血圧変動を感知することができた．

それらの症例の中で，ストレス反応性昇圧に対してウェア

表 2 実験プロトコールの概要

【1】メンタル・ストレス負荷による昇圧
(Mental stress-induced BP elevation)
①「2つの物語に対する暗記（復唱）」負荷
②「計算（暗算）」負荷
1）100 から 7 を連続的に引き算していく
2）3597-59, 1703-17, などの暗算
【2】歩行負荷中の昇圧
(Physical stress-induced BP elevation)
【3】起立による血圧の変化
(Orthostatic hypotension)

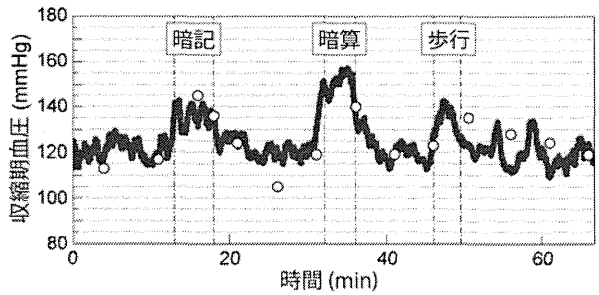

図 2 62 歳女性におけるウェアラブル血圧センサーとカフ血圧
～ストレス反応性昇圧に対する有用性が確認された一例～

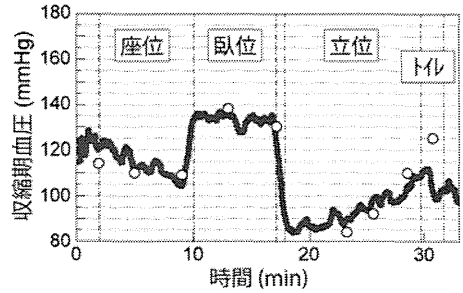

図 3 78 歳男性におけるウェアラブル血圧センサーとカフ血圧
～起立性低血圧に対する有用性が確認された一例～

ラブル血圧センサーの有用性が確認された代表的な症例の血圧測定結果を図2に示す.

カフ血圧測定の収縮期血圧を○印で示す. ウェアラブル血圧センサーによる収縮期血圧の推移は60 beats medianにて表示してある. この症例は認知機能評価としてMini-Mental State Examination (MMSE) 24/30点, Revised Hasegawa Dementia Scale (HDS-R) 26/30点であり, 患者本人の物忘れに対する訴えはあるものの認知機能評価としては軽度認知機能低下(mild cognitive impairment:MCI)のレベルである. 暗記や暗算によるメンタル・ストレス負荷に対して, カフ血圧値は少なくとも20 mmHg以上昇圧していることが分かる. 一方, ウェアラブル血圧センサーによる収縮期血圧の推移を見てみると, そのストレスによる昇圧を再現でき, さらにその昇圧の程度もカフ血圧からの昇圧度から比較するとはるかに大きいことが分かる. 具体的には暗記ストレスによる昇圧はほぼカフ血圧での昇圧の程度と同じであったが, 暗算ストレスにおいては血圧センサーでは約40 mmHg以上上昇していた. 前述したように, カフを用いた血圧測定にはある一定の間隔(ブランク)が必要となる. この結果から見ると, 従来カフ血圧にて連続して測定していても, その間に今までに見えていなかった急峻な血圧上昇や『超』短期変動が存在していた可能性がある.

実際, 認知機能の程度によりカフ血圧の昇圧レベルに差が存在することも報告されており[Kawashima 07], 軽度認知機能低下の傾向にある症例においてはストレスに対して大きな負荷と認識し, 結果的に大きな昇圧が惹起されていることが想定される. 以上より, 生活習慣病としてのリスクを持ち合わせていない高齢者であっても, 従来のカフ血圧測定で描出することができていなかった血圧変動の程度が「認知機能レベル」に大きく依存していることも示唆される. よって, 今後に向けて, この関係を解明すべく症例を重ね検証をすすめていく予定である.

5.2 フィジカル・ストレス反応性の血圧変動の結果

歩行負荷においても, 図2の例においてウェアラブル血圧センサーにより急峻な昇圧(約30 mmHg以上)が確認し得た. カフ測定では歩行中には事実上測定することが不可能である. 特に下半身の筋力低下や脳卒中による麻痺などを持ち合わせている高齢者では, 歩行自体の活動が身体的ストレスになる. その意味でも, 高齢者における身体活動時の血圧変動を確認する上でこのウェアラブル血圧センサーの有用性が伺える. さらに, 収縮期血圧と心拍数から計算されるDouble Productは心負荷レベルを反映しているとされ, 高齢者の歩行リハビリテーションを遂行するにあたり, 過度な心負荷を避ける目的としても良い目安になり得ることが期待される.

また, 認知症に加え起立性低血圧を伴う症例に対して, ウェアラブル血圧センサーにより詳細な血圧変動を同定できた代表的症例を図3に示す.

この症例は認知機能低下に加え自律神経障害も持ち合わせ, 起立性低血圧によりめまい・立ちくらみ, そして転倒を繰り返している症例である. 明らかな脳血管障害は認めないものの, これらの問題に対する恐怖心も併存して, 日常生活における行動範囲が非常に狭められてしまっている. 今回, 起立性の血圧低下に対してウェアラブル血圧センサーを装着し精査を行った. 座位から臥位への体位変換を行っただけでも約20 mmHg以上の血圧上昇がみられ, さらに能動的起立(すなわちSchellong試験)を行ったところ, その起立動作に移っている途中段階から劇的な血圧低下が確認され, 最終的に収縮期血圧が約90 mmHgまで低下した. その後は, ごくわずかな血圧回復を示した.

起立性低血圧をチェックするために, 臨床診療においてはカフ血圧測定を用いてTilt-up試験(受動的起立)やSchellong試験(能動的起立)がよく行われている. この結果から考えると, 測定間隔のあいたカフ血圧値よりも, このウェアラブル血圧センサーによって描出できる詳細な血圧変動の方が高齢者独特の病態を反映する可能性が示唆される.

5.3 考察

以上より, 開発したウェアラブル血圧センサーを血管壁硬化の進んでいる高齢者において臨床応用したところ, 非侵襲的に鋭敏な『超』短期変動を捕えることができた. このウェアラブル血圧センサーをより臨床の場で活用することにより, 従来のカフ圧迫による頻回な苦痛を与えることなく, 様々な環境の変化やストレス下における高齢者の血圧の『超』短期変動を捕えることができると考えられる. 結果に示したように, メンタル・ストレス負荷においてこのセンサーにより昇圧が認められた症例はそれぞれ暗記負荷と暗算負荷で40%と48%であり, また歩行負荷では66%であった. 昇圧が全ての症例において確認されなかった理由として, この50症例には物忘れを主訴とする症例も多く含まれており, 認知機能の低下に応じてメンタル・ストレス負荷が十分かからない症例も存在する可能性が高い. また, 同時にこの対象群には下肢筋力低下も持ち合わせる症例も含まれており, 歩行という動作においては十分な負荷を与えることが限界であった症例も含まれる. 平均年齢80歳の高齢対象者だからこそ, 様々な認知機能や下肢筋力のレベルが存在するが, 認知機能の低下が軽度で留まっている者や歩行が円滑に行える症例においては, 少なくともこの血圧センサーにより超短期血圧変動を感知することができた.

今回の臨床実験を通じて, この血圧センサーを用いての収縮期血圧の絶対値の決定にはキャリブレーションの問題が重要である. 特に血管特性(動脈壁の硬化度など)の個人差が非常に大きな高齢者の場合には, 今回のキャリブレーションの方法ではまだ不完全な部分が残されている. 今後, 同一症例における再現性やbaPWV (brachial-ankle Pulse Wave Velocity)を代表とする動脈壁硬化度とウェアラブル血圧センサーによるデータの相関などの基礎的検討を加えながら, さらに幅広い臨床病態への応用として, 認知機能レベルによるストレス昇圧の反応性の差異, 易転倒性の症例における血圧低下の関与のレベル, さらには高齢者の様々なリハビリテーション時における血圧モニタリングに対するウェアラブル血圧センサーの有用性を検討していく予定である.

カフレスで連続的に血圧を測るセンサーに関して, 脈波伝播速度法を応用した同種の事例がMITより発表されている[McCombie 07]. 一般に測定部の高さが変わると, 水頭圧分の補正が必要になる. このデバイスに関しては, 手首および指でセンスしており, 腕の動作による影響は避けられない. 特に, 歩行中や食事中などの活動状態の際に大きく問題となる可能性があり, 加速度センサーによりこれを補正しようとする論文である. 一方, 本論文で用いた血圧センサーの場合には, 耳たぶという場所を選択しており, 可能な限り体動

の影響を少なくできると考えており，体動の補正なしでも議論が可能である．なお，頭部（耳たぶ）で測ることによる水頭圧の補正は，算出パラメータに含まれるので無視できる．

さて，どちらのセンサーを用いるとしても，臨床の場での十分な活用までにはいくつかの問題を含んでいる．まず1つ目には高齢患者さまご自身の装着感である．現時点でこのウェアラブル血圧センサーを装着した高齢者に聞き取りを行うと，基本的には耳たぶへのセンサーの装着感には大きな問題を訴えてはいない．とはいえ，歩行障害や手指の動作に支障をきたしている高齢者において，いかに身軽な装着として感じることができ，そして体動の影響をいかに少なくできるかが課題であろう．また 2 つ目に，センシング後に算出した血圧値を幅広い医療関係者が見やすい形としていかにリアルタイムに表示できるかも大きな課題である．そこに患者さま側へのデータのフィードバックにも配慮を必要とする．過度な『超』短期変動を捕えることができた時に，その異常をより迅速に診療内容に応用でき，そして患者さま側にも分かりやすい形でデータを共有できるかをさらに検討する必要がある．

6. おわりに

今回，東京大学附属病院・老年病科における高齢症例に対してウェアラブル血圧センサーの有用性を証明すべく，特に『超』短期変動に焦点を合わせて検討した．メンタル・ストレス反応性の昇圧に対しても，そして起立性血圧低下に対しても，カフ血圧値と比較し安定した収縮期血圧の推移を算出することができた．今後，高齢者において，脳・心血管系疾患の発症予防にも「厳格な血圧変動管理」という視点での有用性が期待できるだけではなく，さらに転倒既往や転倒リスクを持ち合わせている高齢者に対しても「過度な血圧変動や過降圧による相対的脳虚血の可能性をチェック」する視点においても非常に有用である．このウェアラブル血圧センサーから得られる情報を臨床診療に活用することにより，最終的には高齢者の日常生活活動度を維持させることにつながることが期待される．

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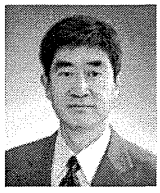

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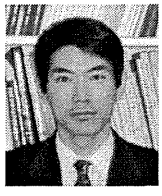

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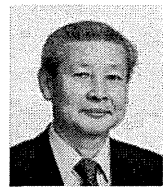

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第54回日本老年医学会学術集会記録
〈シンポジウム8：高齢者糖尿病の管理—J-EDITから得られたもの—〉

3. 高齢者における身体活動量（Physical Activity）の意義

飯島　勝矢

Key words：身体活動，メタボリック症候群，動脈硬化予防，脳心血管イベント抑制，ワーキング
（日老医誌　2013；50：56-59）

はじめに

身体活動度（physical activity：PA）は生活活動強度とも言い換えられ，心血管イベント発症予防および糖尿病や高血圧，脂質異常症などの動脈硬化危険因子の発症予防・進行抑制において相関が言われている．実際，幾つかの疫学研究において，高い身体活動度の集団において心血管イベント発症率および死亡率が低いことが報告されている[1)2)]．しかし，これらのエビデンスは基本的には一般の健康な人々における報告が大半を占め，一般の人々より身体活動度が低下している高齢者において，特に日常生活での身体活動度が及ぼす影響については十分検討されていない．今回，高齢者2型糖尿病患者への介入試験Japanese Elderly Diabetes Intervention Trial（J-EDIT試験）での登録時データを基に，メタボリック・シンドローム（metabolic syndrome：以下MetS）の有無に関して食事摂取カロリーとPAレベルの両面から検討を行った．

高齢者メタボリック症候群と身体活動量

MetSは食の欧米化から過剰な摂取カロリーが大きな問題として取り上げられている．また，高齢化がますます加速し，高齢者における心血管疾患の発症予防を考える上で，高齢者におけるMetSの詳細な検討が必要とされる．すなわち，中壮年層を対象として食事管理（カロリー制限）と運動療法を積極的に指導することは疑いの余地がない方向性であるが，高齢者自体にはたして当てはまるかどうかはまだ議論の分かれるところである．その意味では，今のところ，高齢者における非薬物治療の方向性は統一見解がなく，個々の医療従事者に任されており，十分な解析がされていないのが現状である．

J-EDIT試験に登録された高齢者2型糖尿病患者938名（平均年齢71.9±4.7歳；男447名，女491名）を対象として，登録時に施行されたアンケートを用いて，「Baecke questionnaireによるBaecke（ベッケ）指数[3)]」に基づいて身体活動量をスコア化し算出した．このスコアは3つのコンポーネント（Work activity，Sports activity，Leisure-time activity）から成り立っており，それぞれに対してスコアを算出し，さらにその3つのスコアを合算しTotal physical activity score（TAS：最高スコア15点）を算出した．また，そのスコアに対し4分位（Q1～Q4）に分けた．（詳細なスコア算出内容は割愛する．）それを連続変数とし年齢と性別のみを調整した上で，登録時の各因子（身体測定，血圧，糖・脂質代謝，認知機能評価，うつスケール，エネルギー摂取量，心血管疾患既往など）との関連をロジスティック回帰で評価したところ，以下の結果を得た．

1）TASが上がる毎に，空腹時血糖およびInsulin値が有意な負の関連を，そしてHDL-C値も正の関連を認めた．

2）身体測定ではウエスト周囲径およびウエスト・ヒップ比，そしてBMIにおいて有意な負の関連を示し，特に前期高齢者において顕著に認められた．

3）認知機能スコアよりもうつ傾向を背景とした活動低下が示唆された（GDSスコアと負の関連）．また，老研式ADLと正の関連も認められた．

4）MetS（メタボ）群とnon-MetS（非メタボ）群の比較において，栄養面での摂取カロリー量を算出したところ，TASが上がる毎に総タンパク質および総脂質摂取量は正の関連を示したが，逆に炭水化物や総カロリー摂取量に関してはQ1からQ4までの4分位間で相関を認めなかった．

Learn from the importance of physical activity level in the elderly. How should we encourage and keep it?
Katsuya Iijima：東京大学高齢社会総合研究機構