Low Protein Fish vs Low Protein Animal Diet Enhances the Propensity for Stroke in Stroke-Prone/SHR

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SUMMARY Weanling male and female, stroke-prone, spontaneously hypertensive rats (SHR/SP) were fed: 1) regular commercial rat chow, 2) low protein fish diet, 3) low protein fish diet + 1% saline, 4) low protein animal diet, and 5) low protein animal diet + 1% saline. The blood pressure of all of the SHR/SP rose rapidly reaching 240 mmHg at 90 days of age; blood pressure of low protein fish diet + 1% saline-fed SHR/SP rose most rapidly, reaching levels ranging from 258 to 300 mmHg. All of these animals developed acute strokes by 90 days of age; none of the other diet-fed SHR/SP manifested cerebral damage. The protein poor diets prevented normal growth, caused hypogonadism, and severely reduced pituitary and adrenal gland weights. The low protein diets were stressful causing significantly increased secretion of adrenocorticotrophic hormone and marked increases in triglyceride, free fatty acid, cholesterol, glucose, and B.U.N. levels. The mixed hemorrhagic-thrombogenic cerebral lesions occurred ipsilaterally in the parietal lobe, involved basal ganglia, and appeared in areas of brain tissue nourished by the middle cerebral artery. It is concluded that the inclusion of 1% saline drinking water with a low protein diet of fish tissue origin specifically, was synergistic in enhancing the propensity of SHR/SP rats to develop their genetically-programmed hypertension and stroke.

Stroke, Vol 14, No 4, 1983

With infinite patience and great ingenuity, Okamoto and Aoki succeeded in developing a genetic strain of spontaneously hypertensive rats (SHR) which mimic essential hypertension in man. Okamoto et al. also bred a sub-strain of SHR which develops accelerated, severe high blood pressure and acute cerebrovascular damage which mimics "stroke" in humans, i.e., the stroke-prone SHR (SHR/SP). Destruction of the basal ganglia is purported to appear in 80% of male SHR/SP at 14 weeks of age with an average blood pressure of 240 mmHg. Because of the unusually accelerated rise in blood pressure of male SHR/SP, their average lifespan is only 33 to 41 weeks. Many investigators availed themselves of this intriguing experimental model of spontaneous cerebrovascular disease but enthusiasm was dimmed in the United States by the generalized failure of the purposed stroke-prone rats to manifest spontaneous cerebral damage. The author expended six years of investigation on these stroke-prone animals and found that although they did indeed develop accelerated and severe high blood pressure, not a single case of spontaneous occurring stroke was encountered. Meticulous gross and microscopic examination of hundreds of SHR/SP revealed that these animals were remarkably resistant to the development of those histopathologic changes which usually accompany severe high blood pressure.

Correspondence with Dr. C. T. Hansen, Animal Geneticist, N.I.H. and Dr. Y. Yamori, Isumo, Japan indicated that the SHR/SP raised in Japan continued to have premature cerebrovascular damage of undiminished intensity and regularity. The explanation for the discrepancy between investigators using SHR/SP in the United States vs Japan was said to be that Japanese investigators feed their SHR/SP a protein-poor fish meal, whereas U.S. investigators feed their SHR/SP protein-rich diets of animal origin. The protein-poor fish diet causes worsening of high blood pressure whereas the protein-rich commercial rat chows of the U.S. palliate the onset of high blood pressure. The Japanese investigators asserted that supplementing a low protein fish diet with 1% saline drinking water would accelerate the onset, incidence, and severity of high blood pressure and strokes in SHR/SP. Therefore, the author reinstituted a new colony of SHR/SP and when sufficient progeny had become available, the young SHR/SP were randomly divided into groups fed: regular rat chow containing protein of animal origin, protein-poor diet of animal origin with or without 1% saline drinking water, or protein-poor diet derived from fish (imported from Japan) with and without 1% saline drinking water. The purpose of this experiment was to confirm: 1) whether a protein-poor diet will exacerbate the development of hypertension and therefore the development of strokes in SHR/SP, 2) whether a protein-poor diet of fish vs animal origin is a specific requirement for the appearance of this phenomenon, and 3) whether the addition of 1% saline to these diets is essential to cause exacerbation of the genetically-programmed propensity of SHR/SP to develop spontaneous and acute cerebrovascular damage.

Methods

All of the SHR/SP animals were obtained from brother-sister matings of breeder stock derived from the original SHR/SP strain of Kyoto, Japan kindly provided by Dr. Carl T. Hansen, Animal Genetics Division, N.I.H. All of the breeder stock were fed a regular commercial rat chow diet (Purina) which has a 24% protein content and were given tap water to drink.
When the litters of pups born to these breeders were weaned at 23 days of age, they were randomly selected and arranged in 5 groups of 12 males and 12 females each and were fed the following diets: Group I - regular commercial rat chow (Purina) with 24% protein of animal origin + tap water, Group II - low protein (10%) fish diet + tap water, Group III - low protein (10%) fish diet + 1% saline, Group IV - low protein (8%) animal diet + tap water, and Group V - low protein (8%) animal diet + 1% saline. The low protein fish diet was imported from Funabashi Farms, Funabashi City, Chiba, Japan and the low protein animal diet was formulated by ICN Biochemicals, Cleveland, Ohio. The systolic blood pressure of each rat was recorded every 10 days under light Seconal anesthesia using the Friedman-Freed microphonic manometer and indirect tail-cuff procedure. After 60 days of feeding the special diets, some of the animals began to have spontaneously occurring strokes. At this point, all of the animals were autopsied by instant decapitation.

Blood samples were collected, centrifuged (refrigerated), and assayed for triglycerides, free fatty acids, total cholesterol, glucose, and blood urea nitrogen (B.U.N.) using the automated techniques prescribed for the Auto-analyzer (Technicon Instruments). Adrenocorticotropic (ACTH) in the blood was also measured as an index of pituitary-adrenal activity using radioimmunoassay kits sold by CIS Radiopharmaceuticals, Inc., Bedford, Mass. The brains, heart, and aorta of each animal were examined for gross evidence of vascular disease and degenerative changes. Pertinent organs from each rat were trimmed and weighed, fixed in 10% formalin, embedded in paraffin, sectioned at 3 μm, and stained with hematoxylin and eosin. Statistical analysis of results was performed using a one-way analysis of variance, chi square test, or Student’s t-test.

### Results

#### General Observations

Initially, all of the animals appeared to be normal. After 2 weeks of feeding the low protein animal diet, it became apparent that these animals were not gaining weight normally. These animals appeared lean, growth-retarded, but otherwise healthy and active. When the animals had been consuming the special diets for 64 days (at 86 days of age), those fed the low protein fish diet + 1% saline suddenly began to convulse, leap about wildly in their cages, and minutes later, manifested severe blanching of the eyes, a Horner’s syndrome-like condition, and bilateral or ipsilateral paralysis of the extremities with and without extensor rigidity. Within 7 days of the commencement of these acute "strokes," 100% of the male SHR/SP animals fed the low protein fish + 1% saline diet had experienced a cerebrovascular event; 76% of the females fed the same diet also experienced a stroke during this same period. None of the other animals manifested any untoward signs. All of the animals were autopsied when they averaged 90 ± 5 days of age.

### Blood Pressure

Within 10 days of feeding the special diets, the blood pressure levels of those receiving fish protein + 1% saline was significantly ($p < 0.001$) above the levels of all other animals (fig. 1). Throughout the 60-day course of the experiment, the blood pressure levels of the fish diet + 1% saline-fed SHR/SP continued to rise at an accelerated rate, reaching close to 200 mmHg when these animals were only 60 days old. At 70 days of age, their blood pressures were 240 mmHg and at the time that these animals began having strokes at 90 days of age, their blood pressures ranged from 258 to 300 mmHg (fig. 2). The blood pressure levels of SHR/SP fed the fish diet without saline lagged below those of the SHR/SP fed a regular diet (fig. 1). The blood pressure levels of the SHR/SP fed the low protein animal diet lagged below the SHR/SP fed the fish diet with the addition of saline causing only a small increase in blood pressure (fig. 1). In general, the blood pressure levels of female SHR/SP followed the same course as those shown for male SHR/SP (figs. 1 & 2) except that blood pressure levels of female rats aver-
FIGURE 2. Final blood pressures of male SHR/SP rats fed a normal or low protein diet of fish or animal origin with or without 1% saline drinking water from the time of weaning until 90 days of age. Each column is the Mean ± Standard Error, n = 12.

Changes in Organ and Body Weight

Both the fish and low protein animal diets took their toll in preventing normal growth. The low protein animal diet had the most devastating effect, i.e., the animals in this group were one-third as heavy as those fed a normal diet (table 1). The protein deficient diets were also associated with significantly (p < 0.001) reduced pituitary and adrenal gland size. Despite the fact that all of these SHR/SP animals had severe high blood pressure (a systolic blood pressure above 130 mmHg is considered to be abnormal in the rat), their heart and kidney weights were progressively reduced according to the sparseness of the diet's protein content (table 1). That is, the heart and kidney weights did not reflect the severity of high blood pressure. In keeping with the reduction in body and pituitary gland size, the testes were also progressively reduced in size concomitant with the degree of protein deficiency. All of the above relationships obtained whether organ weights were expressed on an absolute weight basis or on an organ weight:body weight × 100 ratio basis. The organ and body weights of female SHR/SP followed the same trend as shown for males (cf. table 1).

Lipids, Glucose, and B.U.N.

The low protein fish diet induced significant (p < 0.001) hyperlipidemia, i.e., triglycerides, free fatty acids, and cholesterol, hyperglycemia, and increased B.U.N. levels (table 2). The hyperlipidemia and hyperglycemia were greatly exacerbated in those animals fed the fish protein + 1% saline diet (table 2). In direct contrast, animals fed the low protein diet of animal origin with or without 1% saline showed significant (p < 0.001) reduction of their lipid and glucose levels (table 2).

Adrenocorticotrophic Hormone (ACTH)

Circulating ACTH levels were significantly (p < 0.001) elevated in SHR/SP fed the stressful low protein diets (fig. 3). Judging from the blood ACTH levels, the stress potential of the diet appeared to be exacerbated in those fed the fish diet and most severely exacerbated in those animals fed the fish diet + 1% saline (fig. 3). (The blood ACTH levels of female SHR/SP followed the same pattern depicted for male SHR/SP (fig. 3)).

Pathology

At necropsy, the fish diet + 1% saline-treated animals manifested large, ipsilateral, mixed hemorrhagic

| Table 1 Differences in Organ and Body Weights of Male, Stroke-PronelSHR Fed Diverse Diets |
|---------------------------------|--------------------|--------------------|----------------|----------------|--------------------|----------------|----------------|
|                                | Final body wt       | Pit                | Thy               | Adr            | Ht                | Kid             | Testis         |
|                                | gms                 | mgs                | mgs              | mgs            | mgs              | mgs             | mgs            |
| Regular diet                   | 326 ± 9             | 11.0 ± 0.2         | 160 ± 23         | 23.0 ± 1.0     | 1493 ± 21        | 1396 ± 23       | 1697 ± 17      |
| Low protein — fish diet        | 288 ± 9*            | 7.0 ± 0.4*         | 148 ± 14         | 21.2 ± 0.7     | 1463 ± 51        | 1178 ± 36*      | 1502 ± 38*     |
| Low protein — fish diet        |                     |                    |                  |               |                  |                 |                |
| + 1% saline                    | 212 ± 10*           | 5.6 ± 0.6*         | 129 ± 17         | 22.8 ± 0.7     | 1240 ± 53*       | 1154 ± 90*      | 1387 ± 22*     |
| Low protein — animal diet      | 131 ± 9*            | 5.4 ± 0.4*         | 147 ± 13         | 15.6 ± 0.6*    | 911 ± 21*        | 714 ± 13*       | 803 ± 24*      |
| Low protein — animal diet      |                     |                    |                  |               |                  |                 |                |
| + 1% saline                    | 128 ± 6*            | 4.7 ± 0.9*         | 138 ± 11         | 12.0 ± 1.3*    | 631 ± 34*        | 560 ± 18*       | 791 ± 38*      |

Data presented is Mean ± Standard Error; n = 12 animals per group
*p < 0.001, experimental diet vs regular diet
†p < 0.05, experimental diet vs regular diet
TABLE 2  Differences in Lipids, Glucose, and B.U.N. Between Male, Stroke-Prone SHR Fed Diverse Diets

<table>
<thead>
<tr>
<th>Diet</th>
<th>Free fatty acids mg%</th>
<th>Trigly.</th>
<th>Chol.</th>
<th>Glucose</th>
<th>BUN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regular diet</td>
<td>179 ± 16</td>
<td>96 ± 4</td>
<td>89 ± 2</td>
<td>147 ± 2</td>
<td>19 ± 1</td>
</tr>
<tr>
<td>Low protein — fish diet</td>
<td>253 ± 20*</td>
<td>129 ± 3*</td>
<td>101 ± 2</td>
<td>209 ± 3*</td>
<td>24 ± 1*</td>
</tr>
<tr>
<td>Low protein — fish diet + 1% saline</td>
<td>306 ± 19*</td>
<td>346 ± 11*</td>
<td>261 ± 6*</td>
<td>256 ± 7*</td>
<td>28 ± 1*</td>
</tr>
<tr>
<td>Low protein — animal diet</td>
<td>98 ± 5*</td>
<td>81 ± 2†</td>
<td>65 ± 2*</td>
<td>103 ± 3*</td>
<td>19 ± 1</td>
</tr>
<tr>
<td>Low protein — animal diet + 1% saline</td>
<td>96 ± 4*</td>
<td>79 ± 2†</td>
<td>67 ± 3*</td>
<td>111 ± 8*</td>
<td>20 ± 1</td>
</tr>
</tbody>
</table>

Data presented in Mean ± Standard Error; n = 12 animals per group
*p < 0.001, experimental diet vs regular diet
†p < 0.05, experimental diet vs regular diet

and thrombotic space-occupying lesions of the parietal lobe (fig. 4). The damage occurred in areas nourished by the middle cerebral artery. Despite the abnormally high blood pressure levels in all of the SHR/SP animals, their brains exhibited edematous swelling but no damage or necrosis. Only the SHR/SP fed fish protein + 1% saline exhibited thickened epicardial and myocardial arteries, glomerulosclerosis, and extensive fibrinohyalin swelling of the cortical branches of the kidney. Aside from occasional foci of myocardial fibrosis, fatty infiltration of the liver and hyperplastic islets of Langerhans which are characteristic of all SHR sub-strains, no significant histopathology was found in any of the other SHR/SP fed low protein diets. The adrenal glands of all special diet-fed animals were small and red (hemorrhagic) instead of the usual but-ter-yellow (connotes lipid content) color. Microscopically, all of the low protein diet-fed animals showed extensive adrenocortical lipid depletion; the zonae glomerulosae of the fish protein + 1% saline-fed SHR/SP were narrow and totally depleted of lipid. The testes (and ovaries) of the same animals contained inti­mal fibrinohyalin lesions of the medium-sized gonadal arterioles. The growth stunted SHR/SP fed the low protein animal diet were emaciated, and displayed no peri-adrenal, epididymal, or retroperitoneal adipose tissue.

Discussion

These findings confirm the claims of Okamoto and Yamori that feeding a low protein fish diet + 1% saline will cause exacerbation of the genetically-programmed high blood pressure and virtually a 100% incidence of strokes in male SHR/SP. The author found that a protein poor diet derived from animal tissue did not cause exacerbation of the high blood pressure or a single stroke in SHR/SP with or without saline, nor did a protein-poor diet derived from fish tissue (without saline) cause exacerbation of hypertension or brain damage. In this experiment, it was the specific combination of a fish diet + 1% saline which was powerfully synergistic in causing severe high
blood pressure, 100% incidence of strokes, and at an earlier time than observed by Okamoto and Yamori, i.e., at 90 days of age vs 100 days.

Yamori et al. have demonstrated that a high protein diet, e.g., a 50% soybean or 50% fish meat diet, will attenuate the development of severe hypertension and will counteract the adverse effect of salt. The higher the content of amino acids in the diet, the more effective was the diet's ability to counteract hypertension and stroke in SHR/SP. Yamori et al. suggest that a high protein diet causes increased urinary excretion of sodium which counteracts the development of high blood pressure and the adverse effects of excess sodium. Yamori's findings are intriguing since epidemiologists have shown that the unusually high incidence of hypertension and strokes among the Japanese may be related to their high carbohydrate and salt intake (highest in the world), high fish consumption, and low protein intake. In Japan, hypertension and stroke is more prevalent in rural areas than in large cities. Stroke in Japan is not associated with obesity or abnormal lipid metabolism but is best correlated with the high ingestion of soy sauce and salt, rice with miso soup (mix of soybeans, yeast and salt), pickles with tea, and highly salted fish, while the ingestion of apples (high in potassium) is associated with a lower incidence of hypertension and stroke. Japanese patients suffering from acute cerebral damage, like SHR/SP rats, manifest "arterionecro-thrombogenic" strokes vs the "athero-thrombogenic" strokes observed in the United States. The "arterionecro-thrombogenic" strokes do not develop in SHR having blood pressure levels below 240 mmHg; the SHR/SP animals having blood pressures of 240 mmHg and higher develop "arterionecro-thrombogenic" lesions of the cerebral cortex. The degree of hypertension appears to be critical to the expression of cerebral ischemia, cerebral arteriopathy, and stroke.

It is of interest that the author found that feeding a high fat diet to SHR will effectively lower their abnormally elevated blood pressure only to learn that Yamori et al. found that a high-fat-cholesterol diet will also decrease the severity of high blood pressure and the incidence of strokes in SHR/SP. Therefore, extremes in dietary content can cause upsets in homeostasis. It is well established that acute malnutrition is stressful and chronic malnutrition can cause severe impairment of pituitary-adrenal function, i.e., equivalent to hypophysectomy. In the SHR in which the abnormal blood pressure was lowered effectively by a high fat diet, the pituitary glands were atrophic. Thus, hormonal factors played a role in the pathogenesis of hypertension in SHR. Suckling SHR/SP rat pups which were malnourished because they belonged to large litters, developed accelerated and severe hypertension and strokes even though they were provided with an adequate diet after weaning. This indicates that even infantile malnutrition, albeit for a short period of time, can entrap conditions which will interfere with homeostasis and cause the eventual development of severe high blood pressure and stroke in SHR/SP.

Similarly, starvation or a reduced caloric intake will prevent or reduce high blood pressure of SHR suggesting that pituitary failure associated with malnutrition may play a role in homeostatic disequilibrium leading to reduced blood pressure. In the present experiment, it is apparent that the low protein diets, irrespective of animal or fish tissue origin, caused severe growth failure and pituitary, adrenal, and gonadal failure indicative of impaired endocrine function. The author has shown that early hypophysectomy, adrenalectomy, or gonadectomy, as well as the exogenous administration of estrogens and androgens, will either reduce or exacerbate the development of high blood pressure in SHR. SHR/SP subjected to the hormonal demands of repeated breeding manifested increased high blood pressure but myocardial infarction rather than strokes. SHR/SP subjected to chronic treatment with a contraceptive steroid also showed exacerbation of their high blood pressure but no evidence of cerebral damage. However, when weanling SHR/SP were fed the low protein fish diet + 1% saline and given a contraceptive steroid simultaneously, both male and female SHR/SP developed massive strokes well in advance of the 90 days of age observed in the present experiment (to be published). This would underscore the author's suggestion that a low protein fish diet + 1% saline enhances the genetically-programmed propensity of SHR/SP to have strokes and that endocrine factors also play a conditioning role in the expression of the genetically-programmed hypertension and stroke in SHR. An interesting example of the interplay of endogenous factors and genetics is illustrated by the finding that rat pups genetically destined to be normotensive adults but suckled by hypertensive SHR mothers developed hypertension when they became adults.

In the acute sense, a low protein diet constitutes a potent stress stimulus to the pituitary-adrenal axis. The SHR/SP fed the low protein animal diet were secreting levels of ACTH which were indicative of severe stress. The observed hyperlipidemia and hyperglycemia in SHR/SP fed the fish protein diet is most likely due to the extra secretion of ACTH, i.e., the resulting extra glucocorticoid production following ACTH stimulation would exert potent lipid-mobilizing and gluconeogenic effects. Therefore, the greater secretion of ACTH and higher lipid and glucose levels in the SHR/SP fed the low protein fish diet indicates that the latter diet was by far the most stressful in nature. Since the addition of saline to the fish diet caused even greater ACTH release and concomitantly the greatest hyperlipidemia and hyperglycemia underscores the synergistic nature of this combination and suggests that increased pituitary-adrenal activity may have contributed to the increased hypertension and strokes in SHR/SP.

We would agree with Yamori et al. that the strokes in SHR/SP resemble those observed in humans. That is, the preponderance of damage in SHR/SP occurs in the basal ganglia as is often the case in humans. The lenticolesstriate arteries which branch off the middle...
cerebral artery appear to be most consistently afflicted in humans suffering from cerebral ischemia, in the SHR/SP of Japan, and in the author’s SHR/SP animals. The author would agree with Yamori et al. that the large parietal lobe lesions found in these SHR/SP are best described as being of the mixed hemorrhagic-thrombogenic variety. Special emphasis should be made of the fact that only the fish protein + 1% saline-fed animals developed intimal fibroinohyalin lesions of the renal and gonadal arterioles. These intimal fibroinohyalin arteriolar lesions are found in SHR with chronic severe hypertension and in repeatedly-bred SHR with hyperaldrenocorticism. The red-colored hemorrhagic and lipid-depleted adrenal cortices of these low protein-fed animals is further confirmation of the stressful nature of these diets. The narrow and lipid-depleted zona glomerulosae of the fish protein + 1% saline-fed rats suggests that these animals have been secreting extra quantities of aldosterone, specifically. In this connection, the author and others have shown that plasma renin levels are low in SHR while circulating aldosterone levels are elevated and treatment of adrenalectomized SHR with aldosterone will restore their high blood pressure.

Conclusion

These experimental findings indicate that a low protein-calorie diet with amino acids derived from fish tissue specifically coupled with extra salt intake is powerfully synergistic in enhancing a particular genetic constellation inherent in SHR/SP which promotes the rapid development of unusually severe high blood pressure and the expression of acute cerebrovascular damage. This work also suggests that endocrine mechanisms, e.g., pituitary-adrenal-gonadal interaction, may be involved in the expression of this diet-related, animal stroke phenomenon. The similarity of these experimental strokes to human disease and the unexpected relationship of nutrition to the pathogenesis of stroke is most provocative and militates in favor of further research and increased attention by clinicians and experimentalists alike.

Acknowledgments

The author is grateful for the dedication and expertise of E. Domingo, D. Conatser, G. Williamson, G. Heap, and J. Wexler.

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Stroke. 1983;14:585-590
doi: 10.1161/01.STR.14.4.585

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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