Enovid-Induced Exacerbation of the Propensity for Stroke in Low Protein Fish Diet-Fed Stroke-Prone/SHR

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SUMMARY Weanling, male and female, stroke-prone, spontaneously hypertensive rats (SP/SHR) were fed either a regular diet, a low protein diet derived from fish tissue + 1% saline drinking water, or the fish diet + 1% saline + daily injections of 0.1 mg Enovid/100 g bw/sc. After 48 days, the Enovid-treated animals developed acute and lethal strokes characterized by massive thrombonecrogenic lesions of the parietal lobe. The blood pressure of the Enovid-treated SP/SHR rose most acutely. The low protein fish diet was markedly catabolic and caused hyperglycemia, hyperlipidemia, elevated ACTH and beta-endorphin levels concomitant with reduced gonadotrophic function. Treatment with Enovid caused severe exacerbation of all of the foregoing changes. It is proposed that a low protein fish diet + 1% saline will accelerate the appearance of strokes in SP/SHR and that Enovid will enhance this effect through its anti-gonadotrophic activity and ability to stimulate increased pituitary-adrenal secretion.

JAPANESE SCIENTISTS have provided investigators with a most intriguing model of spontaneously developing high blood pressure concomitant with spontaneously appearing strokes, i.e., SP/SHR. Unfortunately, enthusiasm for this experimental model became dampened when U.S. investigators did not encounter spontaneously appearing strokes. The author has established that repeatedly bred rats will spontaneously develop Cushing's disease-like degenerative changes, e.g., hyperglycemia, hyperlipidemia, hyper tension, arteriosclerosis, and accelerated aging where-in the incidence and severity of the degenerative changes parallels the number and frequency of breedings. When SP/SHR were bred repeatedly, they manifested definite exacerbation of the hyperglycemia, hyperlipidemia, hypertension, and other Cushingoid stigmata characteristic of breeder rats but instead of developing strokes, they developed myocardial infarcts. Ancillary investigations of several SHR substrains indicated that the pathogenesis of their naturally occurring hypertension may be due to genetically-programmed abnormal activity of the hypothalamic-pituitary-adrenal-gonadal axis. Therefore, the author treated male and female SP/SHR with a contraceptive drug (Enovid) which is known to cause hypertension, hyperglycemia, hyperlipidemia, myocardial infarction, and strokes in animals and in humans. Although treatment with Enovid did cause exacerbation of the naturally occurring high blood pressure in SP/SHR, none of the animals developed cerebrovascular damage.

Many experimental maneuvers known to exacerbate hyperglycemia, hyperlipidemia, and hypertension were tried in an attempt to elicit strokes in SP/SHR. None was successful. Recently, following the suggestion of the originator of the SP/SHR, the author fed SP/SHR a low protein diet derived from fish tissue (rather than from animal tissue) along with 1% saline drinking water. When these animals became 90 days old, all of them had experienced spontaneous, severe strokes which were lethal. The salient aspect of this earlier experiment was that a low protein diet, specifically of fish tissue origin, coupled with extra salt intake was synergistic in causing exacerbation of the naturally occurring high blood pressure in SP/SHR and concomitantly caused strokes in animals which would not have developed strokes if fed a normal or a low protein diet of animal tissue origin. This low protein fish diet + 1% saline regimen was catabolic, caused hypogonadism, reduced pituitary and adrenal gland weights, but caused significant increased ACTH secretion, hyperlipidemia, hyperglycemia, and elevated BUN levels. All of these hormonal and metabolic conditions can be produced in SP/SHR by the administration of a contraceptive drug. Therefore, male and female SP/SHR were fed the low protein fish diet + 1% saline when they were weaned from their mothers. Some of these animals were injected with the contraceptive drug, Enovid, to ascertain whether the hormonal and metabolic changes induced by Enovid would further exacerbate the stroke-promoting effects of the low protein fish diet + 1% saline.

Methods All of the SHR/SP animals were obtained from brother-sister matings of breeder stock derived from the original SP/SHR strain of Kyoto, Japan provided by Dr. C. 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 normal 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 3 groups of 12 males and 12 females. One group of males and females was fed the normal diet and two groups of males and females were fed the special low protein fish diet + 1% saline. One of the groups fed the fish diet + saline was injected with...
Enovid suspended in saline,\textsuperscript{10} i.e., 0.1 mg/100 gms bw/sc, daily, 5 times per week. The effect of Enovid on male and female SP/SHR fed a regular diet has been established\textsuperscript{10} and was not repeated.

Systolic blood pressure was recorded at 5 day intervals using the Friedman:Freed microphonic manometer and indirect tail-cuff procedure. At 48 days of age, the Enovid-treated animals began to die of acute strokes necessitating termination of the experiment. At autopsy, blood samples were removed from each animal, centrifuged (refrigerated) and assayed for glucose, free fatty acids, triglycerides, total cholesterol, and blood urea nitrogen (BUN) using the automated techniques prescribed for the Auto-Analyzer (Technicon Instruments). In addition, circulating adrenocorticotropic (ACTH) and beta-endorphin were measured by radioimmunoassay using kits provided by CIS Radiopharmaceuticals, Inc., Bedford, Mass., for ACTH, and by Immuno Nuclear Corp., Stillwater, Minn. for beta-endorphin. The brains and pertinent organs of each animal were examined for gross evidence of degenerative changes. Organs were trimmed and weighed, fixed in 10% formalin, embedded in paraffin, sectioned at 3 μm, and stained with hematoxylin and eosin. Statistical analyses of the data were performed using a one-way analysis of variance, chi square test, or Student’s t-test.

Results

General Observations

The fish diet + 1% saline + Enovid-treated animals promptly lost weight despite normal food intake in parallel with quick reduction of scrotal size. After two weeks ingestion of the fish diet, the SP/SHR not treated with Enovid were stunted in growth compared to SP/SHR fed a normal diet. Forty eight days after inception of the experiment, male and female SP/SHR fed the fish diet + 1% saline and treated with Enovid suddenly began to convulse, leap about wildly, and then became prostrate manifesting severe blanching of the eyes, a Horner’s syndrome-like condition, and bilateral or ipsilateral paralysis of the extremities with and without extensor rigidity. These animals died within 42–72 hrs of the onset of the stroke. Since many of the animals in the Enovid-treated group were experiencing acute strokes, the experiment was terminated 50 days after its inception when the animals were 48 ± 5 days old. None of the other SP/SHR manifested any untoward changes.

Blood Pressure

Within 5 days of the onset of the experiment, there was a clear separation of the blood pressure levels of the Enovid-treated animals and the non-drug-treated animals (fig. 1). The blood pressure of the Enovid-treated animals was significantly (p < 0.001) higher than all other groups and the ascent of the blood pressure levels in the drug-treated SP/SHR was progressively steeper culminating at levels (prior to autopsy) significantly (p < 0.001) above all other animals, i.e., ranging from 180 to 192 mm Hg 3 days prior to the appearance of acute strokes (fig. 1). The blood pressure levels of SP/SHR fed the fish diet + 1% saline alone also rose progressively but not as acutely as the Enovid + diet treated animals, culminating at levels considerably above (p < 0.001) those fed a normal diet (fig. 1), i.e., ranging from 146 to 150 mm Hg 3 days prior to autopsy. Blood pressure levels above 130 mm Hg is considered to be abnormal in adult rats. The blood pressure levels of female SP/SHR were consistently lower than those of male SP/SHR irrespective of treatment.

Changes in Organ and Body Weight

The animals fed the low protein fish diet + 1% saline did not grow normally but the body weight of those treated with Enovid + fish diet were significantly (p < 0.001) much more compromised (table 1). The pituitary glands of the experimental animals were also significantly (p < 0.001) reduced in a parallel manner. The thymus glands were involuted in fish diet-fed SP/SHR compared to those fed a regular diet. Ingestion of a low protein fish diet caused adrenal glandular enlargement in male SP/SHR but reduction of adrenal gland weight in female SP/SHR. Enovid appeared to accentuate the effect of the fish diet on pituitary and...
adrenal gland weights (table 1). The heart and kidney weights of the fish diet-fed animals were reduced ($p < 0.001$) commensurate with the reduction of body weight. Treatment with Enovid appeared to accentuate this trend in reduced heart and kidney size. Particularly striking was the severe involution of the testes and ovaries of the animals treated with Enovid ($p < 0.001$) (table 1). All of the above gravimetric comparisons and relationships remained valid whether organ weights were expressed on an absolute weight basis or as the ratio of organ weight/body weight $\times 100$ basis.

**Lipids, Glucose and BUN**

Ingestion of the low protein fish diet caused definite ($p < 0.001$) hyperlipidemia in all animals (table 2). Administration of Enovid did not evoke consistent worsening of the hyperlipidemia. That is, free fatty acid and blood cholesterol levels were clearly elevated ($p < 0.001$) in Enovid-treated SP/SHR vs fish diet-treated animals (table 2) whereas circulating triglyceride levels were lower in Enovid-treated animals vs those fed the fish diet alone. All of the fish diet-fed animals manifested marked ($p < 0.001$) hyperglycemia. Those treated with Enovid manifested the most severe ($p < 0.001$) hyperglycemia. The low protein fish diet caused a moderate increase ($p < 0.001$) in BUN levels (table 2).

**Adrenocorticotropic Hormone (ACTH)**

Circulating ACTH levels were significantly ($p < 0.001$) elevated in all animals fed the low protein fish diet (fig. 2). Enovid caused definite ($p < 0.001$) exacerbation of this heightened ACTH secretion in male and female SP/SHR. (Female rats characteristically secrete greater quantities of ACTH than males under quiescent or stressful circumstances).

### Table 1 Differences in Organ and Body Weights of Male and Female, Stroke-PronelSHR Fed a Regular or Low Protein Fish Diet or Treated With Enovid

<table>
<thead>
<tr>
<th></th>
<th>Final body wt</th>
<th>Pit</th>
<th>Thy</th>
<th>Adr</th>
<th>Ht</th>
<th>Kid</th>
<th>Testis/ovary</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Male</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regular diet</td>
<td>326±9</td>
<td>11.0±0.2</td>
<td>160±23</td>
<td>20.0±1.0</td>
<td>1493±21</td>
<td>1396±23</td>
<td>1697±17</td>
</tr>
<tr>
<td>Low protein fish diet + 1% saline</td>
<td>212±10*</td>
<td>5.6±0.6*</td>
<td>129±17</td>
<td>22.8±0.7*</td>
<td>1240±53*</td>
<td>1154±90*</td>
<td>1387±22*</td>
</tr>
<tr>
<td>Low protein fish diet + 1% saline + Enovid Rx</td>
<td>130±13*</td>
<td>3.5±0.6†</td>
<td>108±36</td>
<td>24.1±1.2</td>
<td>861±67*</td>
<td>752±31*</td>
<td>240±49*</td>
</tr>
<tr>
<td><strong>Female</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regular diet</td>
<td>225±8</td>
<td>12.3±0.5</td>
<td>172±16</td>
<td>29.3±1.0</td>
<td>1046±24</td>
<td>846±23</td>
<td>39±2</td>
</tr>
<tr>
<td>Low protein fish diet + 1% saline</td>
<td>182±4*</td>
<td>9.3±0.6*</td>
<td>98±11*</td>
<td>27.0±0.8†</td>
<td>971±23*</td>
<td>792±18†</td>
<td>28±3*</td>
</tr>
<tr>
<td>Low protein fish diet + 1% saline + Enovid Rx</td>
<td>112±5*</td>
<td>2.4±0.3*</td>
<td>71±9</td>
<td>24.1±1.5†</td>
<td>762±28*</td>
<td>671±27*</td>
<td>9±3*</td>
</tr>
</tbody>
</table>

Data presented is Mean ± Standard Error; n = 12 animals per group.
* $p < 0.001$, fish diet + 1% saline vs regular diet; fish diet + 1% saline + Enovid vs fish diet + 1% saline.
† $p < 0.05$.

### Table 2 Differences in Lipids, Glucose, and B.U.N. Between Male and Female, Stroke-PronelSHR Fed a Regular or Low Protein Diet or Treated With Enovid

<table>
<thead>
<tr>
<th></th>
<th>Free fatty acids</th>
<th>Trigly.</th>
<th>Chol. mg%</th>
<th>Glucose</th>
<th>BUN</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Male</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<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 + 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 fish diet + 1% saline + Enovid Rx</td>
<td>368±21*</td>
<td>308±10*</td>
<td>297±11*</td>
<td>297±13*</td>
<td>28±1</td>
</tr>
<tr>
<td><strong>Female</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regular diet</td>
<td>180±14</td>
<td>92±7</td>
<td>99±2</td>
<td>147±2</td>
<td>22±1</td>
</tr>
<tr>
<td>Low protein fish diet + 1% saline</td>
<td>356±21*</td>
<td>391±16*</td>
<td>239±4*</td>
<td>248±8*</td>
<td>27±1†</td>
</tr>
<tr>
<td>Low protein fish diet + 1% saline + Enovid Rx</td>
<td>406±13*</td>
<td>302±19*</td>
<td>307±14*</td>
<td>288±12*</td>
<td>27±1</td>
</tr>
</tbody>
</table>

Data presented is Mean ± Standard Error; n = 12 animals per group.
* $p < 0.001$, fish diet + 1% saline vs regular diet; fish diet + 1% saline + Enovid vs fish diet + 1% saline.
† $p < 0.05$. 
FIGURE 2. Terminal blood adrenocorticotrophic hormone levels of male and female SP/SHR fed a regular diet, a fish diet + 1% saline, or a fish diet + 1% saline + Enovid from the time of weaning until they became 45 days old. Each column is the Mean ± Standard Error, n = 12.

Beta-endorphin

Radioimmunoassay-reactive beta-endorphin levels paralleled the secretion of ACTH. Beta-endorphin levels were heightened \((p < 0.001)\) in SP/SHR fed the fish diet + 1% saline and were highest \((p < 0.001)\) in those subjected to Enovid treatment (fig. 3).

Pathology

At necropsy, the fish diet + 1% saline + Enovid-treated animals manifested large, ipsilateral, mixed hemorrhagic and thrombotic space-occupying lesions of the parietal lobe. The gross and microscopic pathology of these lesions have been described in detail. \(^{11}\) None of the other SP/SHR exhibited evidence of cerebral pathology. The fish diet + 1% saline + Enovid-treated animals also manifested severe fatty infiltration of the liver, whereas the other animals showed comparatively mild fatty infiltration of the liver. The pituitary glands of the fish diet + 1% saline-fed animals exhibited hyperplasia and deep staining basophilia but no distinction could be made in pituitary gland cytology between those fed the special diet with or without Enovid treatment. Similarly, although the adrenal glands of all of the special diet-fed animals were hemorrhagic and exhibited extensive lipid depletion, especially of the zonae glomerulosae, no distinction could be made between those treated or not treated with Enovid. The ovaries and testes of the Enovid-treated SP/SHR were severely involuted. There was no evidence of histopathology in the hearts and kidneys. The characteristic hyperplasia of the islets of Langerhans in SHR was slightly exacerbated in the fish diet-fed animals with marked degranulation of the insulin secreting beta cells in those treated with Enovid.

Discussion

The most salient feature of this experiment is that although a low protein fish diet + 1% saline will enhance the propensity of SP/SHR to develop acute strokes, the superimposition of treatment with a contraceptive drug will cause significant acceleration of those conditions which favor the appearance of an acute stroke in SP/SHR. Okamoto\(^1\) and Yamori\(^2\) found that SP/SHR fed the fish diet + 1% saline will develop acute strokes when they are 90 days old (males). Wexler\(^11\) found that SP/SHR fed the fish diet + 1% saline will develop acute strokes as early as 60 days of age. It is remarkable that these SP/SHR subjected to a regimen of Enovid + fish diet + 1% saline developed strokes at a significantly earlier time, i.e., 48 days of age.

It is pertinent to emphasize that the author has shown that the capacity to promote strokes in SP/SHR is not due to the low protein content of the diet per se but to some enigmatic combination of factors linking a low protein diet specifically of fish tissue origin and extra saline. The author has shown that a low protein diet of animal tissue origin + 1% saline will not cause strokes in SP/SHR. \(^{11}\) Perhaps the configuration of specific amino acid moieties peculiar to fish tissue is essential for this synergistic effect. Yamori et al\(^{12}\) demonstrated that a high protein diet, e.g., a 50% fish meat diet or a high protein diet from animal meat, will attenuate the development of severe hypertension and

Figure 3. Terminal blood beta-endorphin levels. (Same protocol as Fig. 2).
will counteract the adverse effect of salt. The higher the content of amino acids in the diet, the more effective is the diet’s ability to counteract the severe hypertension which leads to stroke in SP/SHR, i.e., a high protein diet hastens the excretion of sodium and reduces blood pressure. This does not explain why a low protein diet of animal origin + 1% saline failed to induce strokes in SP/SHR.

The prime tenet for the present investigation is the fact that a protein poor diet will interfere with gonadotropin secretion, inhibit normal growth, increase ACTH release but cause reduced pituitary and adrenal gland weight as well as increased BUN levels, hyperglycemia, hyperlipidemia, and hypertension. These hormonal and metabolic changes also follow treatment with contraceptive drugs. The combination of a low protein fish diet + 1% saline and concomitant treatment with a contraceptive drug should accentuate the severity of these hormonal and metabolic conditions and hasten the appearance of acute strokes in SP/SHR. Although this was indeed the case, it is difficult to assign a prime role to any of the conditions which comprise this spectrum of hormonal and metabolic changes. The consensus is that hypertension rather than hyperglycemia and hyperlipidemia is most directly linked with cerebrovascular disease. Within this context, it may well be that the central modus operandi of the fish + saline + Enovid regimen was its ability to accelerate the rise in blood pressure. In the previously reported experiment, it was observed that the arterionecrothrombogenic strokes observed in SP/SHR would occur only when blood pressure exceeded 240 mm Hg. The degree of severity of hypertension appeared to be critical for the expression of cerebral ischemia, cerebral arteriopathy, and spontaneous strokes. In the case of these Enovid-treated SP/SHR, the blood pressure levels ranged only from 180–192 mm Hg 3 days prior to the onset of stroke. This would suggest that the temporal rise in blood pressure relative to the age of the animal is also of prime importance.

Chronic treatment with Enovid has a definite catabolic effect in rats, which mimics the effect of a low protein diet. The combination of Enovid + fish diet exacerbated this catabolic effect which may have aggravated the effect of the acutely rising blood pressure in these young SP/SHR. A low protein diet is akin to a protein diet of animal origin + 1% saline which mimics the effect of a low protein diet of animal origin + 1% saline. The Enovid-treated animals were hemorrhagic and greatly depleted of lipid. This would suggest that this treatment was stressful causing intense stimulation of the pituitary-adrenal axis. This was born out by the unusually high levels of circulating ACTH and beta-endorphin found in fish diet + Enovid-treated animals. The intense fatty infiltration of the liver observed in Enovid-treated animals would also attest to the hyperadrenocorticism in these animals. The normal conjugation and metabolism of steroids would be greatly compromised in fatty livers permitting prolonged steroid circulating time. The hyperglycemia and hyperlipidemia observed in these animals is in accord with the potent gluconeogenic and lipid-mobilizing effects of extra activity of the pituitary adrenal axis. Although it is not clear how the hyperglycemia or hyperlipidemia would contribute to the enhancement of the appearance of strokes, it is clear that increased activity of the pituitary-adrenal axis contributes to increased blood pressure which can be more readily related to cerebrovascular damage.

It is well known that estrogens stimulate increased pituitary-adrenal activity. Recent evidence has confirmed that sex steroids, e.g., estrogens, progesterone, stimulate increased levels of brain beta-endorphin. The estrogen-progestogen composition of Enovid may have caused the marked increase in blood beta-endorphin levels. The greater increase in blood beta-endorphin levels in parallel with greater blood levels of ACTH in fish diet + Enovid-treated animals also comport with the concept that this regimen caused intense stimulation of the pituitary-adrenal axis. It is well established that during stress the secretion of endogenous opiates, e.g., beta-endorphin, parallels the secretion of adrenocorticoids. The author has treated SP/SHR with the anti-opiate, Naloxone, during acute stroke and observed significant prolongation of their usual survival time (to be published). These findings suggest that the endogenous opiate system may be involved in the pathogenesis of or survival of an acute cerebrovascular damaging event.

The severely involuted and atrophic testes and ovaries in the Enovid-treated animals attest to the potent biofeedback effect of this contraceptive agent on the hypothalamic-pituitary-adrenal-gonadal axis. The author has postulated that there may be a relationship between abnormal hypothalamic-pituitary-adrenal-gonadal activity and the pathogenesis of accelerated vascular disease and aging. This hypothesis is pertinent in consideration of the reproductive effort and the development of hyperadrenocorticism or Cushing’s disease. Salmon develop hyperglycemia, hyperlipidemia, hypertension, progeria, culminating in strokes and death as they swim upstream to their spawning grounds. Similarly, repeatedly bred male and female rats develop a Cushing’s disease-like spectrum of abnormal hormonal and metabolic changes in parallel with the number and frequency of breedings. Cushing’s disease in humans often follows parturition and all pregnant women manifest Cushingoid changes wherein the expression of severity of the symptoms is dependent
upon the ability of their liver and proteins to conjugate and convert free steroids into bound steroids, e.g., transcortin. Patients taking contraceptive pills are in a sense pseudopregnant. In the pseudopregnant state, the biofeedback of the estrogen-progesterone combination prevents gonadotropin release but secretion of ACTH, thyrotropic hormone, prolactin etc., is not inhibited. Therefore, Cushingoid conditions appear in women taking contraceptive drugs particularly in those in which protein binding of steroids is deficient. This relative hyperadrenocorticism could explain the occasional appearance of hyperlipidemia, hyperglycemia, hypertension, thromboses, myocardial infarction, and stroke in young women.6,7 Similarly, in these animal investigations, the addition of Enovid to the fish diet + 1% saline regimen may have been synergistic in producing a hyperadrenocorticoic condition in both male and female SP/SHR encouraging the precipitation of stroke in animals genetically programmed to develop strokes.

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References
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