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Influence of short-term repeated fasting on the longevity of female (NZB × NZW)F1 mice

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Abstract

Caloric restriction in rodents is well known to retard the rate of aging, increase mean and maximum life-spans, and inhibit the occurrence of many age-associated diseases. However, little is known about the influence of short-term repeated fasting on longevity. In this study, female (NZB × NZW)F1 mice were used to test the physiological effect of short-term repeated fasting (4 consecutive days, every 2 weeks). The results showed that fasting mice survived significantly longer than the full-fed mice, in spite of the fasting group having a heavier body weight than the control group. Mean survival times for fasting and control mice were 64.0 ± 15.3 and 47.9 ± 9.4 weeks, respectively. Short-term repeated fasting manipulation was also effective on the prolongation of life-span in autoimmune-prone mice.

Keywords: Caloric restriction; Fasting; Autoimmune; Longevity

1. Introduction

Caloric restriction prolongs the mean and maximum life spans and inhibits age-related diseases in rodents (McCay et al., 1935; Fernandes et al., 1978; Kubo et al., 1984a,b, 1987, 1992a,b; Weindruch et al., 1986; Masoro, 1988; Weindruch and Sohal, 1997). Many experiments using this manipulation have been done to test the key concept of 'undernutrition without malnutrition'. Although a number of

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hypotheses have been proposed, the precise mechanism responsible for this effect is not definitely understood. Fasting therapy was first used mainly for the treatment of obesity (Duncan et al., 1963; Drenick et al., 1964; Wing et al., 1983a), however, it has also been notably effective in the treatment of psychosomatic disorders, and is a common treatment for psychosomatic diseases in Japan (Yamamoto et al., 1979). Our experience with patients with allergic or gastroenteric diseases indicates that fasting therapy is very effective. To test our hypothesis that the effects of short term repeated fasting might also influence longevity or disease in rodents, we did an animal experiment using short-lived, autoimmunity-susceptible (NZB × NZW)F1 (B/W) mice, which represent one of several short-lived autoimmune-prone strains. Mice of this strain have been extensively studied as a model of human lupus erythematousus (Theofilopoulos and Dixon, 1986). These mice spontaneously develop autoimmune manifestations including formation of various autoantibodies and also develop a fatal immune complex glomerulonephritis. Profound influences of diet on development and expression of autoimmune disease in some strains of mice have previously been reported (Kubo et al., 1984a,b, 1987, 1992a,b). In B/W mice, life span has been doubled and sometimes even tripled by reduced caloric intake (Kubo et al., 1987), however, little is known about the effects of repeated fasting. In the present experiments, the effect of repeated fasting on body weight, immunological function and longevity is presented.

2. Materials and methods

2.1. Mice

(NZB × NZW)F1 (B/W) mice were bred in our colony and weaned at 6 weeks of age. Specific pathogen-free conditions were maintained throughout the period of this study. The room was operated on a 12-h light/12-h dark cycle at constant temperature and humidity. At 6 weeks of age, female mice were housed in metal cages (five animals per cage) and randomly assigned to one of two groups, a repeated fasting group or a control group fed ad libitum, each group consisting of ten mice for the evaluation of survival, and five mice for the representative immunological evaluation at 26 weeks of age. The mice were monitored until death to establish relative survival times. Dead animals were removed when discovered during daily checks of the cages.

2.2. Fasting regimen

Fasting periods were for 4 consecutive days, every 2 weeks. When fasting was started, the fasting mice had access to only water. Except for these 4 days, the animals had free access to food and water. The control animals were fed commercial lab diet (CLEA rodent diet, Osaka, Japan) ad libitum; the same as the fasting group diet. The composition of the diet was as follows; 8.9% water, 25.4% soybean meal as protein, 4.4% vegetable cooking oil as fat, 4.1% dehydrated alfalfa meal as

fiber, 6.9% crude ash, 50.3% cereals as carbohydrates, minerals and various vitamins. All animals were usually weighed just before the fasting period began. This fasting protocol was begun at 6 weeks of age and continued to death. Representative mice were sacrificed at 26 weeks of age to permit immunological analysis for evaluation.

2.3. Proteinuria

Proteinuria was always assayed just before the fasting period with tetrabromphenol paper (Bayer-Sankyo, Tokyo, Japan) on fresh urine samples. The test is graded $1–4^+$ (1^+, 30 mg/100 ml; 2^+, <100 mg/100 ml; 3^+, <300 mg/100 ml; and 4^+, <1000 mg/100 ml). In this experiment, high-grade proteinuria was designated as $\geq 2^+$.

2.4. Cell preparation for immunologic assay

Mice were bled by cutting the femoral arteries and sacrificed by cervical dislocation. Spleens were collected aseptically. Single cell suspensions were made by gently squeezing spleen tissue between two glass slides in Hanks' balanced salt solution (Gibco, Grand Island, NY), with gauze filtration to remove large residual fragments. Cells were washed three times with Hanks' balanced salt solution before use.

2.5. Culture medium

Added to RPMI 1640 culture medium (Gibco) were 1 μM sodium pyruvate, 5 mM HEPES, penicillin (100 units/ml), 100 μg/ml of streptomycin, 5×10^{-5} mol of 2-mercaptoethanol, and 10% FCS. Normal CBA/H mouse serum (1%) was used instead of FCS to assay responses to mitogen stimulation or mixed lymphocyte reaction.

2.6. Mitogen stimulation

Mitogen-induced blastogenesis was measured using: phytohemagglutinin P ([PHA-P] Difco, Detroit, MI), 0.1% v/v; concanavalin A ([ConA] Calbiochem, La Jolla, CA), 2 μg/ml; *Salmonella typhosa* lipopolysaccharide ([LPS] Difco), 50 μg/ml in RPMI 1640 medium.

2.7. Natural killer (NK) cell activity, mixed lymphocyte reaction (MLR)

Natural killer cell activity, and mixed lymphocyte reaction assay were measured as previously described (Kubo et al., 1992b). Spleen cells of C57BL/6 mice were used for stimulator cells of MLR.

This experiment was reviewed by the Ethics in Animal Experimentation Committee of the Faculty of Medicine, Kyushu University and carried out under the control of the Guidelines for Animal Experiments of the Faculty of Medicine,

Kyushu University and The Law (no. 105) and Notification (no. 6) of the Japanese Government.

2.8. Statistics

Statistical analyses were performed with Student's t-test for parametric data. Survival rates were calculated by use of the Kaplan–Meier's method. For all analyses, the significance level was set at $P = 0.05$. Data analysis was done with Statview Version 5.0 software (Abacus Concepts, Berkeley, CA, USA) on a Macintosh computer.

3. Results

3.1. Growth curves

Mice were of two groups; a fasting group and a control group. The body weight of each group before, during, and after the first fasting time is graphed in Fig. 1. The body weight of the fasting group was significantly decreased as compared to the control group. However, the fasting group gained weight rapidly after refeeding started and became significantly heavier than the control group. Fig. 2 shows the lifetime growth of both groups. Weight was taken just before fasting. The most striking observation was that the average body weight of the fasting group was increased as compared to that of the control group; body weights at 18, 20, 26, 28, 58 weeks were significantly different as indicated in Fig. 2. The fasting mice weighed approximately 10% more than the control mice.

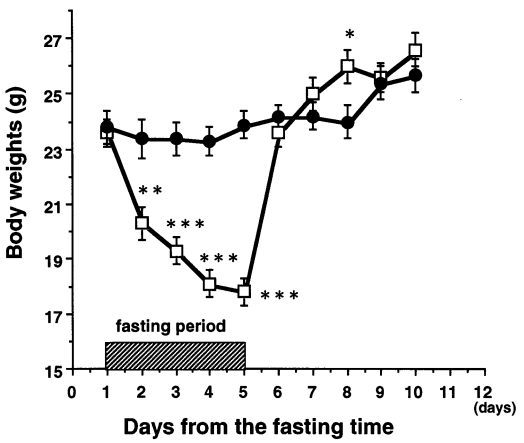

Fig. 1. Body weights of the ● control, and □ fasting groups before, during, and after the first fasting period. The fasting period is shown in this graph as a striped square ▨. Values are mean (g) ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$ when compared with an age-matched ad libitum group.

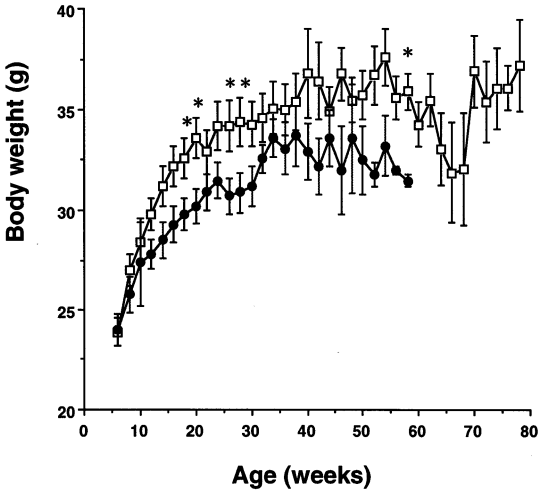

Fig. 2. The life-time growth curves in female B/W mice of the ● control, and □ fasting groups. Weights were recorded at 2-week intervals. Body weights of each group were significantly different at 18, 20, 26, 28 and 58 weeks of age. * $P < 0.05$ when compared with an age-matched ad libitum fed group. The growth curves of control and fasting groups terminated at 65 weeks, and 89 weeks, respectively, with the death of the last mouse. The mice of the fasting group weighed approximately 10% more than those of the control group. Results are shown as mean (g) ± SD.

Table 1
Influence of fasting on various organs in female (NZB × NZW) F1 mice at 26 Weeks of age[a]

Group	Body weight	Spleen ($\times 10^{-2}$)	Liver	Thymus ($\times 10^{-2}$)	Kidney ($\times 10^{-1}$)	Adrenal gland ($\times 10^{-3}$)
Control	32.2 ± 2.6	9.1 ± 2.4	1.5 ± 0.2	4.8 ± 0.6	1.1 ± 0.1	8.1 ± 0.4
Fasting	32.0 ± 1.5	8.1 ± 1.3	1.6 ± 0.1	5.7 ± 0.6*	1.4 ± 0.1**	8.8 ± 0.2**

[a] Short-term repeated fasting effects on body weights and various organs in female B/W F1 mice sacrificed at 26 weeks of age. Five animals were used for each group in this experiment. Each value represents the mean (g) ± SD.

* $P < 0.05$.

** $P < 0.01$, significantly higher than the corresponding control value.

3.2. Organ weights

At 26 weeks of age when both of the groups were on feeding phase, the body weights of the representative mice, which are different mice from the survival group, from each group showed no differences, however, the kidney, thymus and adrenal gland weights of the fasting mice were significantly higher than those of the control mice, as shown in Table 1.

3.3. Proteinuria

Animals of the two groups were also compared with respect to proteinuria. The mice fed ad libitum began to develop severe proteinuria at 22 weeks of age, and all had developed proteinuria by 50 weeks (Fig. 3). The fasting mice, however, gradually developed proteinuria after 30 weeks, and 90% showed proteinuria by 78 weeks of age.

3.4. Mitogen responses

At 26 weeks of age, no significant differences in responses to PHA, ConA or LPS were observed between mice in the fasting and control groups. Fig. 4 shows the results of the influence of fasting on spleen cell response to PHA, Con A and LPS.

3.5. NK cell activity

NK cell activity was not different between the fasting and the control group ($12.5 \pm 2.2\%$ vs. $12.7 \pm 4.3\%$, E:T ratio = 100:1).

3.6. MLR

There was no significant difference in MLR between the fasting and control group (stimulation index $= 4.7 \pm 0.5$ and 3.9 ± 1.1, respectively).

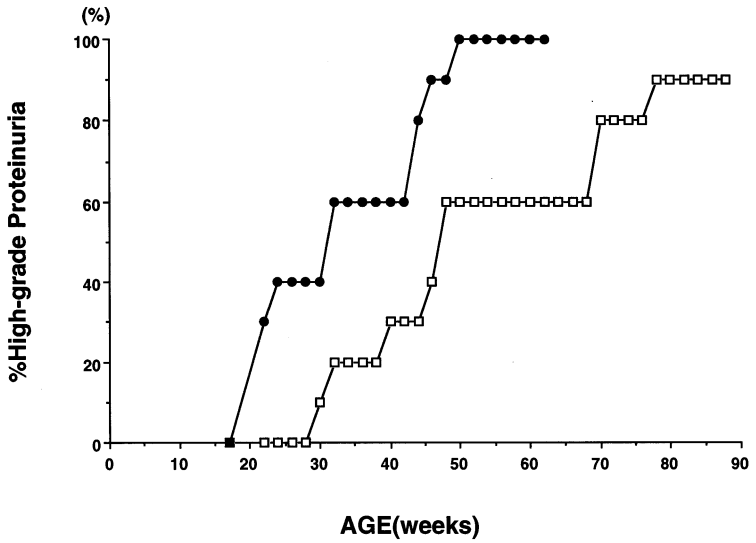

Fig. 3. Effect of fasting on the cumulative progression to high grade proteinuria of female B/W mice over the total life span: ●, control goup; □, fasting group. Proteinuria was assayed just before the fasting period at 2-week intervals. Positive proteinuria was designated as $\geq 2^+$.

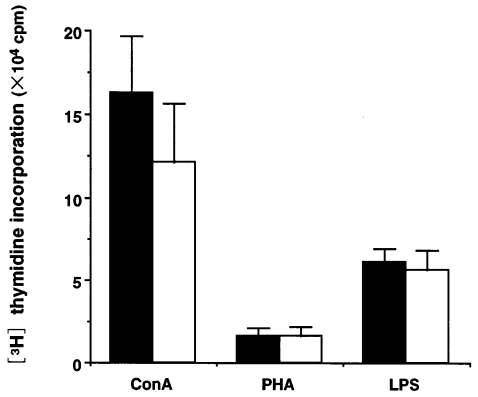

Fig. 4. Comparison of ConA, PHA, and LPS between the control ■ and fasting □ mice at 26 weeks of age. Spleen cells were cultured for 64 h at 37°C and then [^{3}H]thymidine was added for an additional 8 h of incubation. Resutls are mean ± SD of 5 mice.

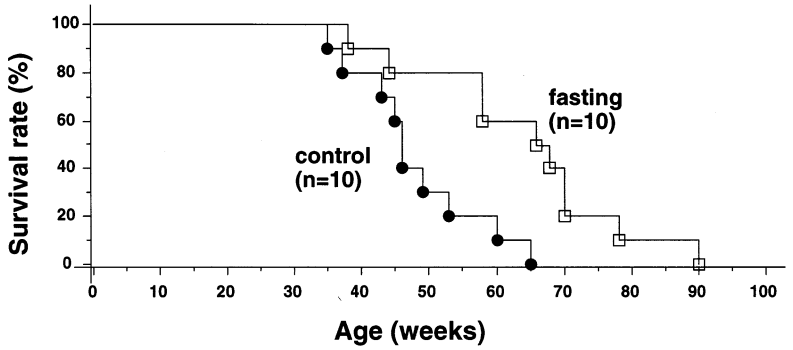

Fig. 5. Survival rate of female B/W mice: ● control group ($n = 10$), and □ fasting group ($n = 10$). The mean times of death (± SD) for control and fasting mice were 47.9 ± 9.4 and 64.0 ± 15.3 weeks, respectively.

3.7. Survival data

Fig. 5 summarizes in graphic form the cumulative mortalities. Although no differences in mitogen response, MLR, or NK cell activity were observed at 26 weeks of age, the mice that repeatedly fasted survived significantly longer than the full-fed mice ($P < 0.005$, with use of the Kaplan–Meier's method). The 50% point of mortality for the control group is 46 weeks, while the 50% point of mortality for the fasting group throughout life is 67 weeks. Mean survival times (± SD) for fasting and control mice were 64.0 ± 15.3 and 47.9 ± 9.4 weeks, respectively.

4. Discussion

The present results show that the female B/W mice that repeatedly fasted had an increased length of the time before disease onset and had moderately prolonged longevity. It has been reported that, in all cases and regardless of calorie source, mice fed 60% of normal caloric intake lived from two to three times longer than their paired full-fed mice (Kubo et al., 1987). The effect of short term repeated fasting on longevity in rodents is not so noticeable as the effect of 'undernutrition without malnutrition'; however, fasting manipulation clearly prolonged the life span of autoimmune mice. Further, surprising to us was the finding that in spite of the prolongation of life and a delay in the development of renal disease, the average body weight of the fasting group was increased compared with that of ad libitum fed mice, which probably indicates that the total caloric intake of the fasting mice was higher than that of the controls. These findings make pressing the question of how fasting manipulation influences mean and maximum life span and how it increases the disease-free interval in these short-lived, autoimmune-prone mice. Numerous hypotheses about the effects of caloric restriction have been proposed, including improved immunological responsiveness (Weindruch and Walford, 1982; Kubo et al., 1984a,b; Walford et al., 1987), the role of high plasma-free corticosterone concentration, the glycation hypothesis (Sabatino et al., 1991; Masoro et al., 1992), the prevention of the age-associated decline in hsp 70 expression (Heydari et al., 1993), the reduction of oxidative damage to macromolecules such as protein, and DNA (Harman, 1956; Sohal, 1993; Sohal et al., 1994; Dubey et al., 1996; Yan et al., 1997). However, few reports are found regarding short-term repeated fasting manipulation. An intermittent feeding regimen (fed every-other-day), somewhat similar to our fasting regimen, was reported by Goodrick and coworkers, however, their studies were not so different from the concept 'undernutrition without malnutrition', in that these animals showed decreased body weights (Goodrick et al., 1982, 1983). We did not find any significant differences in immune function between the fasting and control groups at 26 weeks of age, but the kidney, thymus, and adrenal gland weights of the fasting mice were significantly higher than those of the control mice, which implies that some immunoendocrine system changes might have occurred during and after the short term repeated fasting. Some reports have indicated that various immune-endocrine values changed during fasting. Wing and coworkers showed that mice fasting for 48 or 72 h had increased resistance to the intracellular pathogen *Listeria monocytogenes* (Wing and Young, 1980). They suggested that the increased resistance resulted from enhanced activity of the monocyte–macrophage cell line. We previously investigated the effects of acute starvation on the immune system function of mice, and found that immune function, including phagocytic activity of macrophages and T cell mitogen, was enhanced by a short-period of starvation, but was suppressed by a long-period of starvation (Kubo et al., 1982). The fasting regimen in the present experiment probably has a tendency to enhance the immune function of the mice, but further studies are needed to resolve the optimal fasting period necessary to achieve the maximum possible life-span in the mice. Ehrenfried and coworkers found that an

acute 48-h fast resulted in a marked induction of hsp70 mRNA levels in the stomach of adult rats; these levels rapidly returned to near-baseline levels after refeeding (Ehrenfried et al., 1996). They speculate that hsp70 mRNA elevation in the gut may lead to subsequent increases of Hsp protein, which plays an important cytoprotective role in the gut after an injury or stress. There are few clinical reports with regard to starvation and fasting therapy. Murray and his colleagues who studied famine victims, reported that the nomad populations had a low incidence of clinically significant tuberculosis, malaria, and brucellosis during periods of starvation, however, the incidence of disease caused by these intracellular pathogens increased dramatically after refeeding (Murray et al., 1975, 1976). Fasting therapy was used mainly for the treatment of obese patients in USA; Wing and colleagues who studied various immune parameters in obese subjects before and after fasting, showed that blood monocyte bactericidal activity and NK cell activity were enhanced by fasting (Wing et al., 1983b). On the other hand, it has also been an effective treatment for psychosomatic patients in Japan (Yamamoto et al., 1979). We previously investigated changes in the immunoendocrine system of patients with psychosomatic disorders during fasting therapy (Komaki et al., 1997). Although the total number of lymphocytes decreased during fasting, NK cell activity increased significantly. Plasma cortisol and DHEAS concentrations also increased significantly. The percentage of CD4 cells was negatively correlated with cortisol concentrations during fasting. These findings indicate that fasting affects immune variables such as T cell subsets and NK cell activity, at least in part through changes in adrenal gland-related hormones. These experimental and clinical data indicate that some endocrine–immune–neural changes occurred during the fasting time. Although the precise mechanism is not clear, these endocrine-immune-neural changes may enhance certain functions of the mice defense system and contribute to prolongation of the life span of B/W mice in spite of increased body weight. Extensive further experimental analyses including CD4/CD8 (Fernandes et al., 1997), Th1/Th2 balance (Iwakabe et al., 1998), various cytokines (Ryffel et al., 1994; Chandrasekar et al., 1995; Fernandes et al., 1997; Spaulding et al., 1997), oxidative molecular damage, hsp70, corticosterone, apoptosis (Holt et al., 1998) and leptin (Ahima et al., 1996), as well as behavioral function (Dubey et al., 1996) and cancer incidence (Weindruch et al., 1992) will be required to clarify the role of fasting.

In conclusion, the present report demonstrates that the effects of short term repeated fasting prolongs life spans and inhibits the development of renal disease in short-lived, autoimmunity-susceptible B/W mice in spite of heavier body weight than found in ad libitum fed mice.

Acknowledgements

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