The effects of dietary restriction (DR) on growth, neurobehavior, and reproduction in developing Kunmin mice were investigated in this study. Male and female mice were fed a standard rodent diet ad libitum (control), 80% of control (20% DR), or 65% of control (35% DR) for 3 months. Body weight of DR mice was reduced relative to control except that of females in the 20% DR group (no difference as compared with control group). Learning and memory retention test in a Y maze demonstrated that DR increased learning, but not retention, in male mice, whereas neither learning nor retention was affected in females. The open-field test revealed no difference in exploratory activity in all groups. Reproductive assessment showed that 35% DR retarded the maturation of reproductive function and reduced fertility compared with other groups. Furthermore, both 20% and 35% DR led to a lower level of sperm motility and a higher level of abnormal sperm relative to control mice. These findings indicate that DR does not cause damaging effects on growth and neurobehavior, but imposes a risk to reproductive development events.

**Key Words:** dietary restriction; body weight; reproduction; learning; fertility; sperm; mice.

Dietary restriction (DR) can extend both mean and maximal life span and reduce and retard the incidence of several diseases, including cancer, diabetes, and neurological disorders (Fernandes et al., 1976; Mattson, 2000; McCay et al., 1935; Sohal and Weindruch, 1996; Weindruch, 1989; Weindruch et al., 1986). DR also can attenuate age-related deficits in learning and memory and motor function in rodents (Ingram et al., 1987; Stewart et al., 1989) and improve behavioral outcomes in experimental models of Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, and stroke (Bruce-Keller et al., 1999; Duan and Mattson, 1999; Yu and Mattson, 1999). The mechanisms underlying DR-induced beneficial effects are still poorly understood, but suggested hypotheses include reduction of oxidative damage, retardation of immunologic declines with age, promotion of synaptic plasticity, and induction of stress proteins and neurotrophic factors (Aly et al., 1994; Djuric et al., 1992; Feuers et al., 1989; Koizumi et al., 1987; Mattson, 2000; Meydani et al., 1990; Weindruch et al., 1986).

The majority of efforts have been focused on the beneficial effects of DR on aging, tumors, and life span in the past decades, but little attention was paid to the toxicological effects of DR, especially in developing animals. It is intriguing that several reports showed the physiological, neurological, and behavioral effects of DR in rodents (Duffy et al., 1997; Hubert et al., 2000), but the influence of DR on the reproductive system is still little known. Based on these considerations, we performed this study to assess the effects of DR on reproductive events in developing mice by 20% (mild) and 35% (moderate) restriction of energy intake, but keeping the same intake of protein, fat, vitamins, and minerals to avoid malnutrition (Weindruch et al., 1986). At the same time, we also observed other important parameters, including body weight and neurobehavior.

**MATERIALS AND METHODS**

**Animals and diets.** One hundred and twenty male and female Kunmin mice, all 1 month of age, were obtained from Institute of Wuhan Biotechnology, Wuhan, China. The animals were housed in individual cages and maintained in environmentally controlled rooms (22 ± 2°C and 50 ± 10% relative humidity) with a 12-h light/dark cycle. They were randomly divided into three groups: control (fed ad libitum); 20% DR (fed 80% of control); 35% DR (fed 65% of control). The diets were prepared according to the compositions shown in Table 1. The food consumption in the ad libitum (control) animals was measured daily. The premeasured amount of restricted diets (relative to 80% and 65% of control mice daily consumption) was given daily to the 20% DR and 35% DR animals, respectively. The contents of protein, fat, vitamins, and minerals were adjusted so that intake of these nutrients would be constant in control and restricted mice (Table 1). The food was changed daily to avoid degradation problems. The water was available ad libitum and changed daily. Body weight was determined weekly.

**Neurobehavioral test.** Learning and memory function, which represent a common hippocampus-dependent Y maze paradigm (Riedel et al., 1995; Wetzel et al., 1980), were assessed in a Y maze following 3 months of DR. The Y maze consisted of three equal arms (40 × 15 × 15 cm) with a stainless-steel grid floor. After a 2-min habituation in the maze, the mice were given a foot shock (1.0–1.3 mA) in the start arm; the animal had to escape to the right arm (correct, no foot shock). Entry into the left arm (error) was punished by further foot shock. The male and female mice were given 20 trials, and the error numbers were recorded for each animal for statistic comparisons.

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1 To whom correspondence should be addressed. Fax: (310) 206-9184. E-mail: agwu@ucla.edu
Food Consumption and Body Weight

The food intake, in grams of food consumed, was 80% (20% DR) and 65% (35% DR) relative to control (ad libitum), shown in Table 1. Body weight of DR mice was reduced relative to control, except for that of females with no difference between 20% DR compared with control group (Fig. 1), suggesting that DR retarded body weight gain in developing mice. There were no significant differences in food consumption per gram body weight in all groups of male and female mice (Fig. 2).

Neurobehavioral Test

Learning performance, but not retention, in the Y maze test at 3 months of DR was significantly better in male mice compared with control group (Fig. 3A). No differences in learning and memory retention were found in DR female mice relative to control animals (Fig. 3B). The open-field test did not reveal differences in any of the three parameters, including number of crossings, path length, and number of rearings in all experimental groups (Fig. 4). These results suggest that DR did not lead to abnormal behavior in male or female developing mice.

TABLE 1
Composition of Diets in Each Group

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>0% (control)</th>
<th>20% DR</th>
<th>35% DR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>20.0</td>
<td>25.0</td>
<td>30.8</td>
</tr>
<tr>
<td>Fat</td>
<td>5.0</td>
<td>6.3</td>
<td>7.7</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>65.0</td>
<td>57.2</td>
<td>48.2</td>
</tr>
<tr>
<td>Minerals mix</td>
<td>4.0</td>
<td>5.0</td>
<td>6.2</td>
</tr>
<tr>
<td>Vitamins mix</td>
<td>2.0</td>
<td>2.5</td>
<td>3.1</td>
</tr>
<tr>
<td>Fiber</td>
<td>1.0</td>
<td>1.3</td>
<td>1.5</td>
</tr>
<tr>
<td>H₂O</td>
<td>3.0</td>
<td>2.7</td>
<td>2.5</td>
</tr>
<tr>
<td>Calories (kcal/g diet)</td>
<td>3.8</td>
<td>3.8</td>
<td>3.8</td>
</tr>
<tr>
<td>Percent of food fed</td>
<td>100% (ad libitum)</td>
<td>80%</td>
<td>65%</td>
</tr>
</tbody>
</table>

Note. Values are g ingredient/100 g diet.

The contents of indicated ingredients were adjusted so that intake of these nutrients would be constant.

The food consumption in ad libitum animals (control) and percent of control food consumption in DR groups are shown.
Reproductive Assessment

The first mating test was performed at 1 month with 20 breeding pairs in each group. The percent fertility and percent live pups at 1 month of DR were significantly lower in the 20% DR group than those of control (Figs. 5A and 5B). Even though there was evidence of mating within the 35% DR group, no female mice were pregnant, resulting in no values obtained for all parameters in the 35% DR group during first mating test. At 3 months of DR, a second mating test was done with the same number of breeding pairs within each group. In the second mating test, we obtained values for all parameters in all groups. The percent fertility and percent live pups were significantly reduced in DR groups compared with control animals (Figs. 5A and 5B). No differences were found for weight of live pups in all groups (Fig. 5C).

The average weight (mg) of testis and cauda epididymis was determined, and the sperm count, sperm motility, and morphology in male mice were evaluated at the end of the 3-month DR experiment. Results showed that the sperm count was significantly decreased in both DR groups relative to control (Fig. 6A). A similar reduction of sperm motility was found (Fig. 6B). The abnormal sperms were dramatically increased in the DR group compared with control, showing a greater effect in the more severely restricted group (Fig. 6C). The average weight of testis and cauda epididymis in restricted groups was not significantly different from that in control animals (Fig. 7).

DISCUSSION

Our results in this study demonstrated that DR reduced body weight gain, consistent with previous reports (Weindruch et al., 1986), and improved learning performance in male mice, but reproductive assessment suggested that DR imposes a risk to reproductive events in developing mice.

The DR regimen in this study, including different restriction levels of energy intake (0% as control, 20% DR, and 35% DR) with consistent intake of essential nutrients in all groups, was based on previous evidence. It has been suggested that the profound antiaging effects of DR are associated with optimi-
zation of restriction regimens (Weindruch et al., 1986). A 30 to 50% restriction of dietary energy intake (without malnutrition), rather than any particular nutrient, can strongly lower the incidence of most spontaneous and induced tumors, delay their onset, and extend the life span of rodents (Weindruch, 1989; Weindruch and Sohal 1997). The beneficial effects of DR appear to depend on restriction of energy intake with adequate intake of essential nutrients (Weindruch et al., 1986). Therefore, optimal nutrient composition and feeding strategies for DR experiments such as levels of restriction, intake of essential nutrients, and term of restriction, are very important for designing experiments associated with DR. Based on these con-

FIG. 4. Open-field test in male and female mice. In each group, n = 10. Number of crossings, path length, and number of rearings are shown. The data for path length were converted to percent of control and presented in bar figure. Values represent mean ± SE.

FIG. 5. Mating test with 20 breeding pairs within each group at 1 month and 3 months of DR. The parameters include (A) percent fertility, (B) percent live pups, and (C) weight of live pups. Values represent mean ± SE. *p < 0.05; **p < 0.01 compared with control.
considerations, we introduced three levels of energy intake (0%, 20%, and 35% DR) with equal intake of fat, protein, vitamins, and minerals to developing mice to find out whether there is a dose (DR level of energy intake)-dependent response. The body weight was reduced in DR mice compared with control group, which is in agreement with other reports, suggesting a retarded gain of body weight with a dose-dependent tendency of reduction in males (Fig. 1). The DR regimen (restriction of energy intake without malnutrition) in this study is different from the reduced food intake commonly encountered in toxicology studies. The reduced food intake caused by a toxicant may lead to decreased intake of all essential nutrients, which then may result in malnutrition and weight loss. Further, our DR regimen is also different from short-term fasting or food deprivation. The latter may lead to malnutrition, subsequently causing pathological or dysfunctional status such as reduced detoxification in liver in mammalian systems (Grattagliano et al., 2000; Shimizu and Morita, 1992).

In this study, we found that DR increased learning, but not retention, in male mice. No effects of DR on learning and memory in females were found. The exploratory activity in an open-field test was unchanged in all groups, similar to results obtained by Ingram et al. (1987). The difference between the study of Ingram et al. (1987) and ours is that they maintained female C3B10RF1 mice on a control or restricted diet from weaning, and tested learning and exploratory activity at 11–15 or 31–35 months of age in control and restricted animals. In that report (Ingram et al., 1987), DR was shown to prevent age-related decline in learning performance in female C3B10RF1 mice, reflecting the beneficial effects of DR on cognition in aged animals. No similar results observed in our study may be due to age- or sex-dependent differential response to the DR regimen. The beneficial effects of 3 months

![Sperm count graph](image)

**FIG. 6.** Sperm evaluation in male mice at the end of 3-month DR experiment. The sperm samples were taken from the right cauda epididymis. In each group, n = 10. (A) Sperm count. (B) Sperm motility. (C) Abnormal sperms. Values represent mean ± SE. **p < 0.01 compared with control.

![Testis weight graph](image)

**FIG. 7.** Average weight of testis and cauda epididymis in control and restricted mice at the end of 3-month DR experiment. In each group, n = 10.
of DR in male, but not female, mice suggested that DR might induce differential effects in male and female animals. The mechanisms remain to be further established, especially the roles of hormone regulation. Our results from neurobehavioral tests indicated that DR for 3 months did not produce damaging effects on neurobehavior in developing mice.

However, reproductive performance and sperm evaluation revealed that DR imposed a risk to reproductive events in developing mice. In this study, first mating test was performed at 1 month of DR (relative to 60 days of age). All values except weight of live pups were lower in the 20% DR group compared with control, but no values were obtained for all parameters in the 35% DR group. The results of a second mating test at 3 months of DR showed that all parameters except weight of live pups were significantly decreased in DR groups relative to control, with a greater effect in 35% DR animals. These results indicated that 20% DR caused mild effects on reproductive function, whereas higher levels of DR (35%) resulted in severe effects on fertility ability. The health and development of pups were affected when their dams were subjected to DR at an early age, resulting in decreased percent of live pups in DR groups. It was reported that fertility was decreased in adult Swiss CD-1 mice by feed restriction for up to 21 weeks (Chapin et al., 1993). However, the mating test was performed by mating DR mice to nonrestricted partners in that study, and the degree of decreased fertility was different from our results. The retarded sexual maturation by DR was also observed in rats (Merry and Holehan, 1979), but the restriction regimen with the same nonenriched diet in both restricted and control animals was different from ours in this study.

Sperm numbers and motility are crucial for normal fertility in developing animals (Chapin et al., 1997; Olds-Clarke, 1988). Motility is a necessary function for sperm transport through the male and female reproductive tract (Olds-Clarke, 1988). In this study, sperm count and motility were significantly reduced in the DR groups compared with control, suggesting that DR affects the normal growth and development of sperm; the mechanisms are unclear. There is evidence indicating that the development of the normal sperm head is polygenically controlled and the increased numbers of sperm with abnormalities reflect the genotoxic effects of prooxidants on germ cells (Wyrobek et al., 1983a). The abnormalities of sperm were reported to be associated with mutation of Y chromosome-linked genes (Krzanowska, 1976; Styrna et al., 1991). The increased abnormalities of sperm observed in this study suggested that DR might lead to genotoxic damage in germ cells. A more severe level of DR elicited higher levels of abnormal sperm, showing a dose (DR level)-dependent response (Fig. 6C). Our results also suggest that DR affects normal growth and development of sperm, resulting in decreased sperm motility and numbers, which may consequently contribute to reduced fertility (Fig. 5). There is a high correlation of sperm abnormalities with mutagenicity in germ cells (Bruce et al., 1974; Topham, 1980; Wyrobek et al., 1983b; Wyrobek and Bruce, 1975). Therefore, our results suggest that DR may have potential mutagenic effects on germ cells in developing animals.

In conclusion, our results indicate that DR retards body weight gain, but does not cause damaging effects on neurobehavior in developing mice. However, DR imposes a risk to reproductive events, including retarded fertility ability, decreased percent of live pups, lowered sperm count and motility, and increased abnormal sperms, the mechanisms of which need to be further investigated. Our findings in this study provide important insights into the toxicological implications of DR and call for further attention to the reproductive system when applying DR regimens to developing animals for basic research or bioassays, or to humans for clinical trials of therapeautic strategies.

ACKNOWLEDGMENTS

We are grateful to Profs. Xiping Zhang and Jiyao Wang for their excellent assistance. This work was supported by funding from National Natural Science Foundation of China.

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