Dietary Cystine Ameliorates Defects in Spermatogenesis via Testosterone Production Induced by Protein Deficiency and Darkness in Rats

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Summary Nutrition and light-dark cycle influence rat testicular development. With 9% casein diet (low protein diet) under normal 12 h-12 h lighting cycles (9P), juvenile rat testes undergo normal growth. On the other hand, a low protein diet with constant darkness (D9P) results in a growth arrest of rat testes. Supplementation of cystine to the low protein diet under constant darkness (D9PC) had a tendency to increase testes weight, suggesting an improvement in growth suppression. Whether the growth suppression of testes in D9P is associated with suppression of spermatogenesis has not yet been shown. We aimed to determine the effect of a low protein diet and constant darkness with or without dietary cystine in testes using a histological technique. In the histological assessment, D9P testes showed a decreased number of seminiferous tubules with elongated spermatids, indicating a functional testicular defect in this group. However, cystine supplementation resulted in enhanced spermatogenesis versus control animals (D9PC vs. D9P) implying the importance of cystine to testicular development in this condition. Furthermore, serum testosterone concentration was increased in D9PC suggesting contribution of testosterone to ameliorate spermatogenesis. From these results, we conclude that cystine supplementation to a low protein diet under constant darkness promoted an increase in testosterone which in turn benefitted spermatogenesis.

Key Words cystine, rat, spermatogenesis, testis, testosterone

Disturbed rhythm by night shift work has negative influences in circadian rhythm and hormonal-dependent metabolism (1–4). On the other hand, protein-energy malnutrition influences circadian rhythm particularly as a disturbance in sleep-awake cycle (5, 6). Dysregulation in circadian rhythms and nutrition can assert various dysfunctions.

To model such dysregulation, light cycle disturbance (7, 8) or malnutrition (9, 10) in relation to rat testis development have been reported. Our group has reported that male rats with low protein diet under constant darkness show less growth in the testis compared to low protein diet under a normal lighting condition (12 h light/12 h dark) (11–13). Also, we have previously reported that adding cystine to low protein with constant darkness condition rescues testicular growth suppression (11); however, the precise mechanism(s) of how low protein diet and constant darkness arrest testicular development and how cystine-dependent phenotype reversal occurs, are still not clear.

In the current paper, we determined spermatogenesis and testosterone production in rats with or without cystine supplementation to low protein diet under normal or constant darkness conditions.

Materials and Methods

Animals. Fischer strain (F344) male rats (n=100, 3-wk-old) were purchased from Charles River Japan, Inc., Yokohama, Japan, and acclimated for 5 d on the AIN-93G diet, which consists of 20% casein and 0.3% cystine supplementation (14). Rats were then divided into four experimental groups, n=24 or 26 per group. The first group (9P) received normal lighting (12 h light/dark cycle) and a low protein diet containing 9% casein. The second group (9PC) received cystine-supplemented diet. The third group (D9P) received constant dark condition with 9% casein diet. The fourth group (D9PC) received constant darkness and cystine-supplemented diet. Details of utilized diet is shown in Table 1. Rats kept in the constant darkness were handled under red lamp in order not to disturb dark condition. Food and distilled water were provided to rats ad libitum. This study was conducted in accordance with the guidelines established by the Japanese...
Table 1. Composition of diets. (g/100 g diet)

| Ingredients                  | Diet groups       |
|------------------------------|-------------------|
|                              | 9% casein (9P)    | 9% casein + cystine (9PC) |
| Milk casein                  | 9                 | 9                      |
| i-Cystine                    | 0.135             | 0.135                  |
| α-Corn starch                | 64.25             | 64.11                  |
| Sucrose                      | 10                | 10                     |
| Cellulose fiber              | 5                 | 5                      |
| Soybean oil                  | 7                 | 7                      |
| Mineral mixture\(^1\)        | 3.5               | 3.5                    |
| Vitamin mixture\(^2\)        | 1                 | 1                      |
| Choline bitartrate           | 0.25              | 0.25                   |
| tert-Butylhydroquinone       | 0.0014            | 0.0014                 |

\(^1\) AIN-93G mineral mixture containing 67.2 g/kg of phosphorus.
\(^2\) AIN-93 vitamin mixture.

Results and Discussion

Groups receiving cystine supplementation (9PC, 149.7±7.3 g and D9PC, 143.5±9.4 g) showed significantly heavier body weights (b.w.) than their counter parts (9P, 120.0±5.1 g and D9P, 118.3±6.5 g), respectively, suggesting a nutritional value of cystine. The testis weight was decreased in dark conditions D9P and D9PC (1.38±0.50 g and 1.53±0.41 g, respectively) compared to their counter parts. 9P and 9PC (1.80±0.20 g and 2.1±0.20 g, respectively). Between D9P and D9PC, D9PC testes weight had tendency of increased mean value than D9P. B.w.-adjusted testes weights also showed no difference between D9P and D9PC (1.16±0.40 and 1.06±0.25 g/100 g b.w., respectively, \(p=0.243\)). This result (experiment period between February and March) is in contrast to our previous report (experiment period between June and July) that testicular weight in D9P was larger than D9P (11). It is not clear why the discrepancy occurred, but one might speculate importance of seasonal matter in conducting sexual organ development study (15).

Microscopically, we observed elongated spermatid containing seminiferous tubules (Fig. 1A, C and G as representatives). Some seminiferous tubules did not have elongated spermatids (Fig. 1E). We found that under normal lighting conditions (9P, Fig. 1B; 9PC, Fig. 1D), testes weights and percent spermatid positive seminiferous tubules are high, which made them distributed in upper right portion of the graphs. On the other hand, constant dark conditions (D9P, Fig. 1F; D9PC, Fig. 1H), resulted in a wider value distribution. By setting a threshold at the minimum percent of spermatid positivity in normal light conditions (77.6%), we divided the plots to over threshold (spermatid developed) and under threshold (spermatid underdeveloped). In D9P, 65% of rats were placed as over threshold and 35% were placed as under threshold (Fig. 1F). With cystine supplementation, over threshold had 85% and under threshold did 15% (Fig. 1H, D9PC). D9PC had tendency of better spermatogenesis than D9P (\(\chi^2\) analysis, \(p<0.109\)). Furthermore, comparing the means of percent spermatid positive seminiferous tubules, D9P was significantly lower than 9PC (\(p=0.002\)), whereas D9PC was similar to normal light condition groups (9P and 9PC) (Fig. 2A), suggesting spermatogenesis restoration in D9PC. Morphologically, Leydig and Sertoli cells showed no difference among groups that cystine effect under constant darkness and low protein diet seems specific to spermatid formation.

As testosterone is a key factor for spermatogenesis, we measured serum testosterone concentrations (Fig. 2B). Among four conditions, there were significant difference (Kruskal-Wallis, \(p=0.046\)). D9PC had higher testosterone among the groups (\(p=0.016\) vs. 9P, \(p=0.014\) vs. D9P). Moreover, per time plot of serum testosterone concentration revealed that rhythm of testosterone production is lost in D9P compared to normal light conditions 9P and 9PC in which peak of testosterone occur at 8:00 (zeitgeber time 1 as light turns on at 7:00) (Fig. 2C, D) which is similar to what have been reported on...
male rats (16, 17). It is noticeable that D9PC group gained serum testosterone rhythm, in which the peak occurred at 2:00 (circadian time 19) (Fig. 2D).

Nevertheless, we observed an improvement in spermatid development in D9PC compared to D9P. This implies that: (1) 9% casein (low protein) diet is not insufficient for rat testicular development when light exposure is present, (2) constant darkness or not having light exposure adversely affects developing testes with low protein diet, and (3) adding cystine to low protein diet with constant darkness restores, at least in part, arrested testicular development. It is reported that cysteine

Fig. 1. Reduced spermatogenesis in condition D9P is attenuated in D9PC. Representative appearances of PAS stain from 9P (A), 9PC (C), D9P (E) and D9PC (G) are shown. Insets show elongated spermatids with red/pink acrosome (arrowheads). Scale bar show 50 μm. Percent spermatid positive seminiferous tubules and testes weight were plotted for 9P (B), 9PC (D), D9P (F) and D9PC (H). Each dot represents individual rat. Percentage of rats over and under threshold are shown in the graph.
Cystine Effect on Spermatogenesis in Rat

Cystine addition to casein-based low protein diet increases plasma cystine about two-fold (18). In a low protein diet, testicular cystine may be reduced from normal amount (19), which would be necessary to produce cysteine-rich protein, protamine that packages chromatin into sperm head (20). Under the normal lighting condition, cystine addition to low protein diet did not increase plasma testosterone (9PC) nor the rhythm. As reported previously, under constant darkness, free-run period of rats become longer than 24 h (21, 22). The rhythm shift of testosterone in D9PC may reflect it. If an increase in testosterone is due to cystine addition alone, then, 9PC should present the increase. However, it occurred only in D9PC. We think that this implies major involvement of light entrainment in 9P and 9PC. Comparing to this, without light stimulation, food intake entrainment takes over, and with unknown mechanism, it may influence hypothalamus-pituitary-gonadal axis to increase testosterone production in Leydig cells.

The exact mechanism that cystine increases serum testosterone in low protein diet with constant darkness is unclear, that molecules influenced by cystine supplementation should be studied in the future study. Our results may imply a possible mitigation in spermatogenesis suppression within people who have disturbances in their daily rhythms.

Disclosure of state of COI
No conflicts of interest to be declared.

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