The Impacts of Photoperiods on Hypothalamic Proteins in the Reproductive Activities of Golden Hamsters

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ABSTRACT : The reproduction of animals is a means to maintain their species. The golden hamsters are seasonal breeders, and their reproduction is regulated by the photoperiod. The shifts of the sexual functions imply the changes of the protein expression, and those are reflected by the proteome. Thus the present study was to examine via two dimensional polyacrylamide gel electrophoresis (2d PAGE), the physiological changes and the alterations of protein expressions in hypothalamus upon the reproductive situation related to the pineal gland. Among the pineal intact animals, the reproductive functions were sustained in animals housed in long photoperiod (LP) but regressed in animals housed in short photoperiod. And those pinealectomized animals showed high sexual activities regardless of photoperiod. Ultimately they were branched into dichotomy, sexually active and inactive animals. Apart from the changes of physiological parameters upon the reproductive conditions, there were obvious differences in proteins extracted from the hypothalamus. The hypothalamus of LP animals presented high levels of enzymes which are involved in the production of energy, glycolysis and Krebs cycle. The increased energy might be related to the GnRH synthesis in hypothalamus and indirectly to the constant cell divisions in spermatogenesis. Taken together, the impacts of the photoperiodic information controlling reproduction could be observed through 2d PAGE. Therefore, the present results suggest the potential of biomarkers collectively to diagnose the fertility and the infertility by way of proteomics in organs with regard to the reproductive system, further could be applied to diagnose various diseases.

Key words : Reproduction, Photoperiod, Pineal gland, Protein, Hypothalamus.

INTRODUCTION

Small mammals in temperate climate are mainly influenced by ambient environmental factors fluctuating with the seasons. Golden hamsters have developed a strategy of reproduction in which their sexual activities are active at summer and inactive during winter weather (Pierpaoli et al., 1996; Stetson & Watson-Whitmyre, 1986; Stetson & Watson-Whitmyre, 1984). They experience torpor at winter like hibernation in the case of bear. It is a behavior that they regularly wake up and get out for sensing the temperature at an interval of two or three days. They give birth to offsprings at a favorite period of time that they can survive and themselves as well. This way of reproduction is regulated by the photoperiod, which is a time of light in a day, and the outcomes can be induced by artificial lights in a laboratory. Regardless of natural light or manmade illumination, the information of photoperiod is transmitted into the reproductive endocrine system, and that is mediated by melatonin secreted by pineal gland upon photoperiodic cues. The photoperiodic information is no longer exerted in the animals with the removal of pineal gland.

This sort of seasonal reproductive breeders undergo various changes, such as light period in a day, temperature, rainfall, and availability of food. Usually a major element present in the environment governs reproduction of animals. Among many seasonal factors from natural environment, photoperiods determine reproductive functions of golden hamsters in which photoperiod can be relatively accurately predicted in a period of one year but other environmental factors can not be anticipated like photoperiod (Reiter, 1980).

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Under the artificial light regimens, the sexual functions of golden hamsters are promoted or maintained when the day time is equal to or over 12.5 hours in a day but are suppressed when the day time is no longer more than 12 hours in a day (Elliott, 1976). At a same line of context, exposure to 24 hours of lights sustains the sexual activities but 24 hour darkness regresses it. Thus daily 12.5 hours of lights is a dichotomy period at which the reproductive activities are divided into active and inactive phases. But there are small differences of the lighting lengths of a day that promote or sustain the reproductive functions at various animals (Stetson & Watson-Whitmyre, 1986; Stetson & Watson-Whitmyre, 1984).

When the golden hamsters matured sexually in long photoperiod (LP) are transferred to short photoperiod (SP), the functions of the gonads are regressed on both sexes of animals. There are no detectable spermatozoa with tails in seminiferous tubules of males, and no overt matured Graafian follicles within the ovaries of females (Gaston & Menaker, 1967). The proestrous surge of gonadotropins, the follicle stimulating hormone (FSH) and luteinizing hormone (LH), which is typical on the estrous cycles of females, can not be witnessed (Donham, et al., 1990). The males with reproductively inactive activity also have remarkably reduced FSH and LH (Choi, 1996; Jackson, et al., 1984). It has been well established that the synthesis and release of the two gonadotropins are determined by gonadotropin releasing hormone (GnRH) from the hypothalamus. Thus the lowered levels of the gonadotropins showing involuted sexual functions by the exposure to SP indicate the diminished release of GnRH, and ultimately result in degeneration of testicular functions (Pickard & Silverman, 1979). That is, SP exerts its effects on the hypothalamus, and suppresses the secretion of GnRH. Therefore, the information of photoperiod that influences the hypothalamus-hypophyseal-gonad axis established well in mammals is eventually estimated to closely related to the hypothalamus invoking the expression of GnRH gene (Smith et al., 2006; Lee & Choi, 2005).

Meanwhile, the reproductive activities are sustained or promoted on both sexes, regardless of photoperiod, in the case of surgical removal of pineal gland (Stetson & Watson-Whitmyre, 1986). The proper administrations of melatonin lead to regression of reproductive function on the other side of treatment. Thus it is generally accepted that the impact of photoperiod affecting sexual activities is mediated by the melatonin. The amount of melatonin in the blood is under the regulation of photoperiod. Melatonin is synthesized and released at night only without lighting (Rollag et al., 1980). But when the animals are exposed to a lighting at night, levels of melatonin are abruptly dropped into the levels of day time. Thus the period of darkness in animals exposed to SP, in comparison to that in animals exposed to LP, lengthens relatively and elongates time period of synthesis and release of melatonin, as a consequence, is appeared that the reproductive functions are involuted. In the end, although it is thought that melatonin exerts its effects on hypothalamus, but there is little study about working proteins in the hypothalamus.

These changes of the endocrine system continue with the imprinting in the living things while they reflect both daily and seasonal changes in the course of evolution over a long time period, and so the changes act as a sort of merit in maintaining of the species of golden hamsters. Along with the recent discovery of the structure of the genome, investigation of proteins should be inevitably followed. Therefore, the present study was to examine the relationship among proteins in relation to the reproductive activities in males of golden hamsters, for understanding the aspects of changes of all kinds of proteins by applying the method of 2d PAGE through the alterations of sexual functions by photoperiod and pineal gland.

MATERIALS AND METHODS

1. Breeding of Golden Hamsters

Male golden hamsters (*Mesocricetus auratus*) used in this experiment were maintained in animal breeding boxes

within the animal breeding room. They were adults, an age of 8 week old or more. The condition of management of animals was approved by the Yongin University Institutional Animal Care and Use Committee (YUIACUC, 2009-01). The animal breeding boxes are manufactured with wood, the period of time of lighting was controlled by the timer, external lighting was blocked completely, and ventilation was equipped with the fans. As with the photoperiods, LP consisted of light of 14 hours and darkness of 10 hours, SP light of 10 hours and darkness of 14 hours. the point of middle time in day time of each photoperiod was synchronized. Food and water were provided *ad libitum* and sanitary management was checked repeatedly.

2. Experimental Groups and Removal of Pineal Gland

Golden hamsters were divided into 4 groups as indicated below. The grown-up adult golden hamsters experienced the period of acclimation for 2 weeks, divided into two groups in which animals in one group were subjected to the surgical removal of pineal gland (pinealectomy, PinX) and animals in the other group were subjected to that kind of surgery without removal of pineal gland. The animals in each group were again divided into two groups and housed in LP or SP for 10 weeks. Body weights were measured from the beginning of the experiment at regular intervals and at the matching time after turning on lights in both LP and SP. Weight of testes were converted from the volume of the testis that was calculated from the measurements of the major axis and the minor axis (Watson-Whitmyre & Stetson, 1985).

Group	Photoperiod	Treatment	Remark
1	LP	None	LP, 14L:10D SP, 10L:14D
2	LP	PinX	
3	SP	None	
4	SP	PinX	

The pinealectomy of hamsters was performed by anesthesia using pentobarbital (25 ㎎/㎏ of body weight) and ketamine (50 ㎎/㎏ of body weight). When they were completely anesthetized, their head was fixed on the stereotaxic apparatus by using ear bars, and their skin covering skull was excised about 5 ㎜ in the middle of head. The skull of the animal was perforated on the position of lambda by the dental drill equipped with the circular saw and the fragment of the skull was kept for later covering. The bleeding was eliminated with cotton while the pineal gland was excised by forceps. The perforated part of the skull was covered with the excised circular form of skull, followed by suture of the skin. Some antibiotics was treated on the excised part and the animals had recovery period of time of 2 weeks, and finally they were moved into each photoperiod.

3. Determination of Testes Weights and Histological Examination

1) Determination of Testes Weights

The volume of testes was measured by laparotomy. As indicated above, after the animals were completely anesthetized, the major axis and the minor axis of testes were measured by vernier calipers after excising the skin overlying the scrotal sac and protruding the testes within the scrotal sac. The skin excised was sutured with surgical thread. The weights of the testes were calculated by the measurements. At the end of the experiment, the testes extracted were kept in freezer (−80℃) until use for histological examination. And testicular index was expressed as percentage by a formula shown below, to consider the relationship of the removal of pineal gland to the changes of body weights.

Testes weights(g)/Body weight(g)×100

2) Histological Examination

Histological examination was performed by using the cryostat (Bright instrument Co., Ltd). The testis kept frozen

(−80℃) was left within the cryostat for more than 30 minutes. The testis was mounted with the embedding medium (Sakura Finetek USA Inc.). The testicular tissue was sliced at 4-6 ㎛ thickness in the animals housed in SP and 12-14 ㎛ thickness in LP, and the slices were laid on the slide glasses. Immediately the slide glasses were put on the slide warmer heated previously into 55℃ for 30 minutes. After the slide glasses submerged in distilled water for 2 minutes, they were stained with hematoxylin (Sigma) solution for 1 minute. In order to remove excess staining solution, the tissue slides were washed with distilled water for 5 minutes and new distilled water for 2 minutes. After the slides were stained with 0.8% eosin Y (Sigma) solution for 30 seconds, they were washed in a series of alcohol of 50%, 70%, 80%, 90%, 95% for 1 minute each, then 100% alcohol twice for 1 minute each. The remained impurities were eliminated by treatment of xylene twice for 2 minutes. The slides were dried entirely to evaporate xylene components and subjected to permanent preparat with canada balsam (Sigma), and finally observed under microscope. The presence and absence of matures spermatozoa, the thickness of the seminiferous tubules, and various cells within the tubule were observed and compared with respect to the reproductive functions.

4. Blood Samplings

The blood was withdrawn by cardiac puncture from the animals, if required, at day time and night time. The blood sampling at night was performed under dim red lighting. Blood was collected periodically from the beginning of the experiment. The whole blood was left at refrigerator (4℃) for at lesat 8 hours, and serum was gained by spinning down. The serum was kept at freezer (−20℃) before use.

1) Measurements of Hormones

After melting the frozen (−20℃) serum, the concentrations of the each hormone were measured by enzyme-linked immunosorbent assay (ELISA, USCNK). The standard hormones were reconstituted with standard diluent and kept for 10 minutes at room temperature (FSH: 40, 20, 10, 5, 2.5, 1.25, 0.625 mIU/㎖, LH: 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78 mIU/㎖, Melatonin: 1,000, 500, 250, 125, 62.5, 31.2, 15.6 pg/㎖). The control tube with standard diluent is the blank as 0 mIU/㎖ of hormones. The amount of 100 ㎕ each of dilution of standard, blank, and samples were added into the appropriate wells in 96 well plates. The plates were covered with the plate sealer and incubated for 2 hours at 37℃. The liquid was removed from each well. One hundred ㎕ of detection reagent A working solution was added to each well and incubated for 1 hour at 37℃ after covering it with the plate sealer. The solution was aspirated and each well was rinsed with wash solution and left it sit for 1-2 minutes. Following snapping the plate onto absorbent paper, the remaining liquid was completely removed from all wells. This procedure was repeated three times. After the last wash, any remaining wash buffer was removed. The plate was inverted and blotted against absorbent paper. The Detection reagent B working solution 100 ㎕ was added to each well and the plate was incubated for 30 minutes at 37℃ after covering it with the plate sealer. Each well was aspirated and washed for 5 times as mentioned above. The 90 ㎕ of substrate solution was added to each well and the plate was covered with a new plate sealer and incubated for 15-25 minutes at 37℃. The plate was protected from light. The liquid will turn blue by the substrate solution. Then 50 ㎕ of the stop solution was added to each well and mixed by tapping the side of the plate. Then the plate was subjected to the microplate reader and conducted measurement at 450 ㎚ immediately.

5. Two Dimensional Polyacrylamide Gel Electrophoresis (2d PAGE)

1) Preparation for Proteins

(1) Tissue Extraction

The golden hamsters were administered to euthanasia by instantly cutting head. At night, they were sacrificed under

the dim red light. Immediately using scissors, both lateral parts of the skull of the head were severed along with the temporal bone (from occipital bone through temporal bone to frontal bone) and top portion of the head was turned over in direction of forward. Then the exposed brain was turned over in direction of backward. If optic nerve was still connected, it was cut at close proximal site to brain. The protruded hypothalamus in the center on the bottom of the brain was excised by bended small scissors, put it right away on dry ice, and frozen. The frozen tissues were kept at −80℃ until use.

(2) Hypothalamic Protein Extraction and Measurement

The hypothalamic tissue kept frozen was excised, weighed, and placed in a tube. The sample preparation solution (4 times weight of hypothalamus) containing protease inhibitor mix (EDTA free, GE Healthcare) was added to the tube. The tissue was grinded with homogenizer (Fisher Scientific, Power Gen35) for 5 seconds and left on ice for 20 seconds. This step was repeated 4-5 times. The sample was transferred to the microcentrifuge tube and the tube was spun at 15,000 rpm for 2 hours. The supernatant was moved into new microcentrifuge tube. The precipitant from the 2-D clean up kit (GE Healthcare) was added, mixed, and left on ice for 15 minutes. And the co-precipitant was added to the tube. The tube was mixed slightly and spun at 12,000 rpm for 5 minutes. The supernatant was decanted and the remained liquid was completely removed. The co-precipitant was again added to the tube and the tube was left on ice for 5 minutes. After centrifugation, the supernatant was removed.

The distilled water was added to the pellet and the tube was stirred until suspension. The wash buffer and the wash additive were added and the tube was stirred for 20-30 seconds and left on ice for 10 minutes. This step was repeated at least 3 times. After spinning at 12,000 rpm for 5 minutes, the supernatant was decanted and the remaining liquid was completely removed after spinning down shortly. The precipitate was dried at room temperature. The sample preparation solution was added to the dried pellet. The amount of proteins extracted was measured in duplicate by Bradford method and the proteins were aliquoted and stored at 4℃.

2) Strip Re-swelling

DeStreak™ Rehydration solution (GE Healthcare) was melted at room temperature. This solution and IPG buffer (GE Healthcare, PH 3-11 NL) were added to microcentrifuge tube, mixed slightly, and spun slightly. IPG box (GE healthcare) was placed on flat position and leveled. The multi-reswelling tray was places on the box and mixed solution was dropped on one site of a groove. The thin film over the gel was peeled off from (−) side of the strip. The gel was contacted with the solution. The lid of the box was closed and the box was allowed to proceed for at least 10 hours.

3) IEF(Isoelectrophoresis Focusing)

The protein extracted (0.2 ㎎) and DeStreak™Rehydration solution melted at room temperature were mixed to make total volume of 100 ㎕. IPG buffer that is 0.5% of total volume was added and mixed slightly and spun down shortly. The IPGphor (GE Healthcare) that was turned on 30 minutes ago was again leveled and equipped with manifold, and the gel of the strip was placed toward the ceiling. The wick paper (electrode pad) submerged with distilled water was placed with overlapping with 5 ㎜ at

Step		Volt (v)	Time
s1	step	100	1:00 Hr
s2	step	200	1:00 Hr
s3	step	300	1:00 Hr
s4	step	500	1:00 Hr
s5	grad	1,000	1:00 Hr
s6	grad	8,000	1:30 Hrs
s7	step	8,000	55,000 Vhr
s8	step	50	10:00 Hrs

both (+) and (−) ends of the strip. The white gold strip of the electrode was positioned to overlap the wick papers placed at both end electrodes. The sample cup was equipped and the cover fluid oil (GE Healthcare) was dropped on the sample cup. The IPGphor program (IEF Program Setting, 13 ㎝, pH 3-11 NL) was set as follows.

4) Polyacrylamide Gel Electrophoresis

12.5% acrylamide solution was prepared by mixing 30% acrylamide+bisacrylamide solution, 4×resolving gel buffer, 10% SDS, 10% APS, and TEMED. This acrylamide solution was poured into glass plate, isopropanol was immediately added. The strip with complete IEF was incubated for 15 minutes in the solution of equilibration solution and DTT. After the gel was formed, isopropanol was decanted and completely removed by using distilled water. The strip with completion of equilibration was lightly submerged in the distilled water and placed closely on the gel, and agarose sealing solution melted right before was added to the strip. The glass plate was mounted in electrophoresis kit (SE 600 Ruby system) and the kit was run at 100V for 1 hour and then at 150V for 4 hours.

5) Silver Staining

The gel that completed the process of electrophoresis was immersed with swaying in the fixing solution (30% ethanol; ethanol 150 ㎖, glacial acetic acid 50 ㎖) for 60 minutes. This step was repeated twice. The gel was transferred to the sensitizing solution and subjected to sway for 120 minutes. The gel was washed with distilled water for 8 minutes 5 times and silver solution was added and left for 60 minutes. The gel was washed with distilled water for 1 minute 4 times and was allowed to the developing solution for 2-5 minutes. The gel was moved to stop solution and allowed to react with swaying for 45 minutes. Then the gel was washed with distilled water for 30 minutes twice.

6. Statistical Analysis

Statistical analysis was performed using Student's *t*-test, if necessary, using analysis of variance (ANOVA). Data were expressed as means±SEM and p value <0.05 denoted the statistically significant difference.

RESULTS

The changes of body weights of the animals in each group were measured to check the health conditions because of a long term experiment spanning 10 weeks. During the entire period there were no behavioral differences between the animals with and without pineal gland. The initial body weights at the beginning of experiment increased gradually with time, but the significant alterations of body weights at the given time point were not detected (data not shown). But there was a tendency of increasing of body weights in intact pineal animals housed in SP.

The reproductive functions of golden hamsters were determined by photoperiod. Among the animals with intact pineal gland, animals housed in LP persisted on the active sexual activities but animals in SP showed completely regressed testes in 10 weeks (Fig. 1). These effects on SP were disappeared in the animals without pineal gland. All animals resulted in dichotomy, animals with active and inactive reproductive functions. The mean testicular weights were reduced into 0.5 g in animals housed in SP with intact pineal gland, indicating testicular regression, thus showing no matured spermatozoa within seminiferous tubules. The other animals demonstrated remarkably thick seminiferous tubules and plenty of matured speramatozoa. Testicular index showed the same results as the absolute testicular weights (Fig. 1).

The levels of melatonin in animals with and without pineal gland are shown in Table 1. Melatonin concentrations were low at day time and high at night in the animals with intact pineal gland regardless of photoperiod. This sort of increase of melatonin at night was reduced into the levels of day times without respect to photoperiod when the animals were pinealectomized. Thus it is thought that pineal

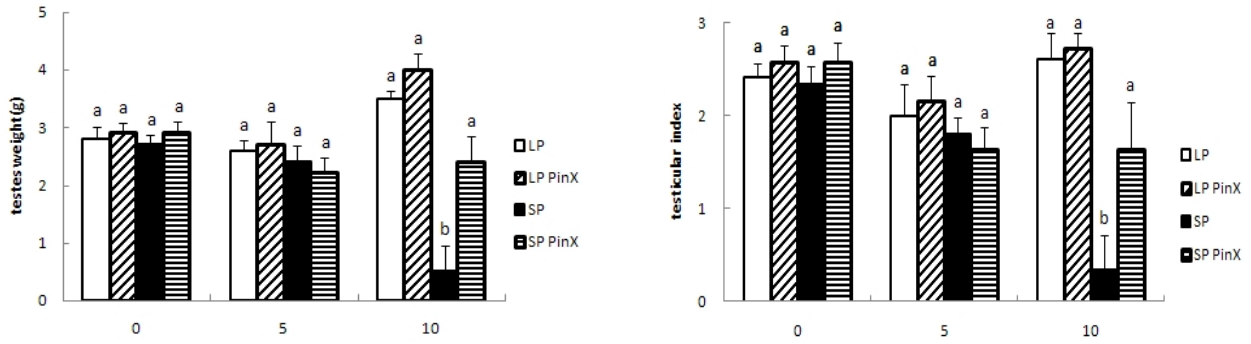

Fig. 1. Changes of testicular weights. The major and minor axis of a testis were measured at pointed time, and converted into weights by a proper formula. In order to understand the relationship of testicular weights to body weights, the testicular index was applied, which is testicular weights divided by body weights and multiplied by 100. The testes of the animals housed in SP showed regression on both cases in 10 weeks. The abbreviations are referred to Table 1. Values are means±SEM (n≧3). Different letters indicate a significant difference (p<0.05).

gland transmits the effects of photoperiod into reproductive endocrine system, and regulates sexual activities.

From these two sorts of animals with reproductively active and inactive style, the former showed all kinds of cells involved in spermatogenesis, such as spermatogonium, primary and secondary spermatocytes, spermatids, and spermatozoa, the latter little cells, spermatogonium, and primary and secondary spermatocytes (Fig. 2). The area of section of seminiferous tubules is diminished into more than one fourth in reproductively inactive animals compared to the reproductively active animals.

In animals whose reproductive activities were regressed, the gonadotropins FSH and LH levels in blood were significantly decreased in 5 weeks (Fig. 3). The concentrations of FSH and LH at the beginning of the experiment were put on week 0. In 10 weeks of the experiment, the both FSH and LH concentrations tended to increase but without significance.

There were some alterations of protein expression upon

Table 1. Melatonin concentrations in blood

	LP(pg)	LP PinX(pg)	SP(pg)	SP PinX(pg)
Day	25.1±0.03^a	33.0±0.38^a	28.1±0.10^a	32.1±0.24^a
Night	62.2±0.20^b	40.2±0.35^a	58.7±0.19^b	31.8±0.13^a

Abbreviations: LP; long photoperiod. LP PinX; animals with removal of pineal gland and housed in long photoperiod. SP; short photoperiod. SP PinX; animals with removal of pineal gland and housed in short photoperiod.
Values are means±SEM (n≧3).
Different letters indicate a significant difference (p<0.05).

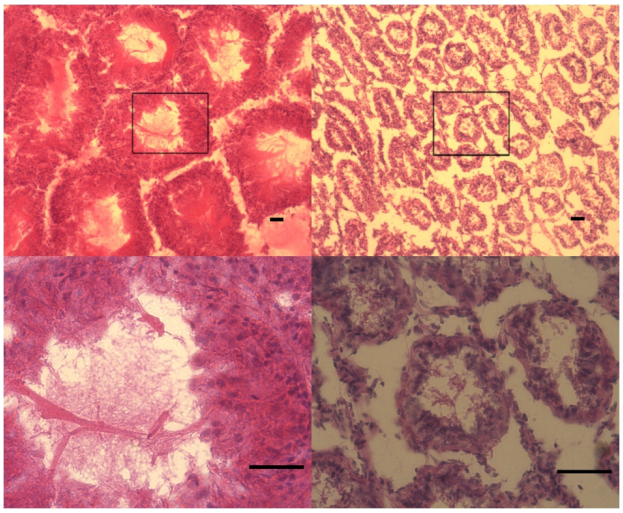

Fig. 2. Spermatogenesis. These are testes of animals with reproductively active and inactive functions. The small rectangular insets in upper panels are magnified at lower panels. The testes at lower left corner shows active spermatogenesis, with thick wall of seminiferous tubules including matures spermatozoa, while the testes at lower right corner inactive spermatogenesis, with thin wall of seminiferous tubules including no matures spermatozoa. upper panels; 100×. lower panels; 400×.

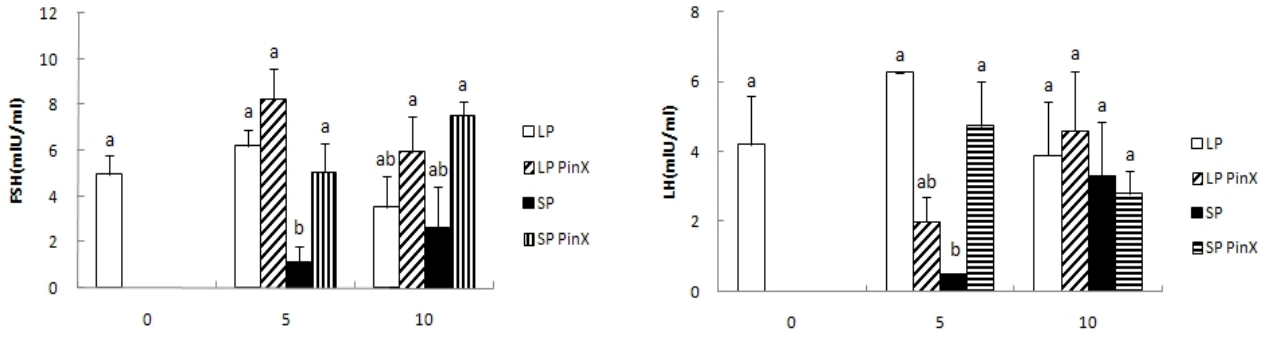

Fig. 3. Blood levels of gonadotropin, FSH and LH. The blood was withdrawn by cardiac punctureand and collected into serum following clotting at refrigerator (4℃) for at least 8 hours. The levels of FSH and LH in SP animals were reduced in 5 weeks, which is a typical phenomena preceding the involution of testes. The abbreviation are referred to Fig. 1. Values are means±SEM (n≧3). Different letters indicate a significant difference (p<0.05).

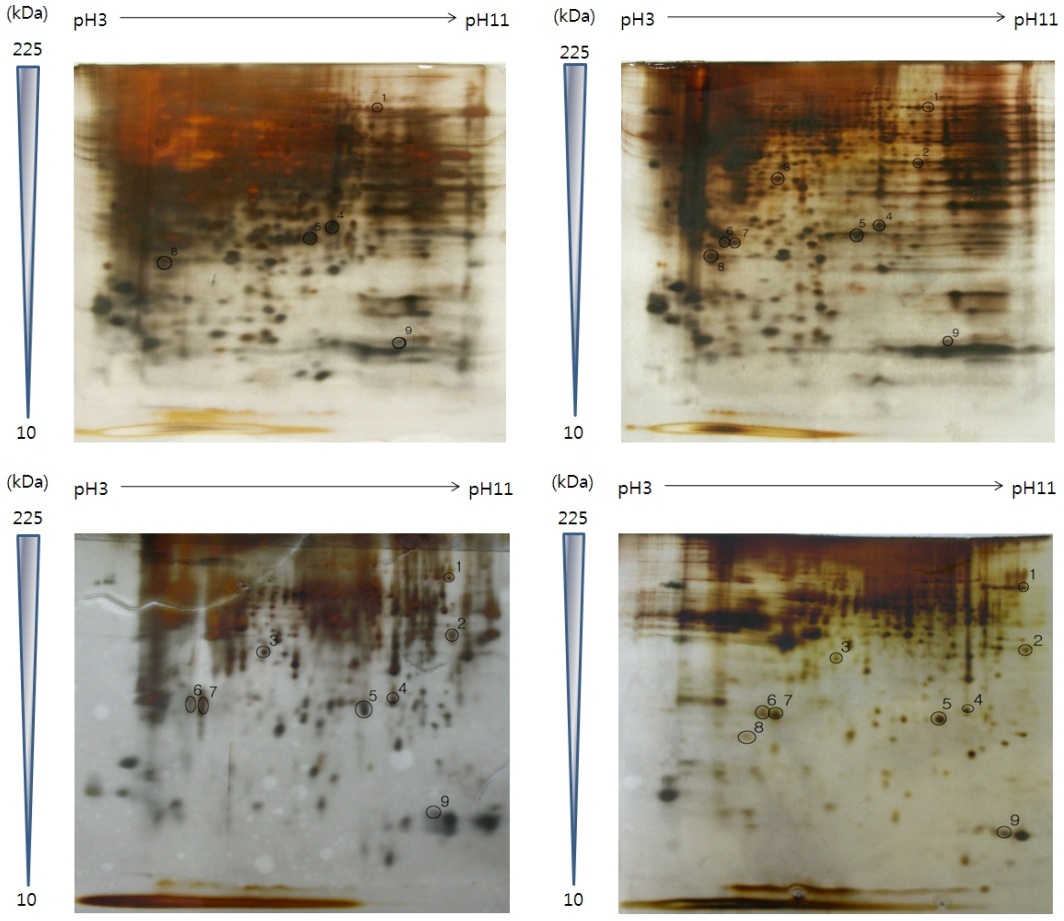

Fig. 4. Representative pictures of hypothalamic proteins by 2d PAGE. The impacts of photoperiod regulating reproduction were observed through silver staining on the 2d PAGE. Large amount of proteins were loaded (upper panels) to detect the substances with lower molecular weight and small amount of proteins were loaded (lower panels) to detect the substances with high molecular weight.

Table 2. Identified hypothalamic proteins on 2d PAGE

No	Predicted MW(Da)	Peptide sequenced	Protein name	LP/SP	Accession
1	85,410	· IVYGHLDDPANQEIER · SQFTITPGSEQIR · NAVTQEFGPVPDTAR · WVVIGDENYGEGSSR	Aconitase 2, mitochondrial	−/−	CAC11018.1
2	46,999	· LYPPSAEYPDLR · SGYFDER · SFLIWVNEEDHTR · GWEFMWNER	Creatine kinase	+/−	EDL79988.1
3	39,588	· APIQWEER · IAEFAFEYAR	Isocitrate dehydrogenase 3(NAD^+) alpha	+++/−	EDL95544.1
4	36,454	· FVEGLPINDFSR	Cytosolic malate dehydrogenase	++++/−	AAA37423.1
5	26,679	· FFVGGNWK	Triosephosphate isomerase	++++/−	NP_075211.2
6		· XGQLQWR · AEAPQPWPR	Similar to retinoic acid-induced protein 1	−/−	Q60465
7	24,766	· LGVAGQWR · NEAIQAAHDSVAQEGQCR · MPFPVNHGASSEDSLLQDAAK	Ubiquitin carboxyl-terminal hydrolase PGP9.5	−/−	BAA01541.1
8	10,926	· KEGGLGPLNIPLLADVTR · QITVNDLPVGR	Peroxiredoxin 2	−/−	Q8K3U7
9		· VNADAVGAEALGRLLVVYPWTQR	Beta major globin chain	−/−	ABU63208.1

· Parts of amino acids sequenced. −, detected. +, degree of detected proteins(The more signs, the more dark spots).

reproductive situations in the 2d PAGE of hypothalamus (Fig. 4). As integrated in Table 2, the influences of photoperiodic information regulating reproductive state were reflected by the proteins. Their roles are elucidated below.

There were many spots of hypothalamic proteins fluctuating up or down upon reproductive conditions. The proteins that had been previously identified with those established in mouse and rat are numbered in Fig. 4.

The number 1 was aconitase, which is an enzyme converting citric acid into isocitric acid, an interim substance in Krebs cycle, resulting in synthesis of ATP as an energy. The proteins numbered 2 was creatine kinase found usually in muscle, which acts to produce ATP in the movement of muscles. The activity of this protein is a little higher in males than females and augmented by exercise. The third spots were isocitrate dehydrogenase 3(NAD^+) alpha, catalyzing isocitrate into α-ketoglutarate and CO_2 while converting NAD^+ into NADH.

The cytosolic malate dehydrogenase was the 4th protein that catalyze malate into oxaloacetate in Krebs cycle, resulting in NADH production. The fifth protein was triosephosphate isomerase that transforms reversibly dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate in glycolysis, essentially resulting in energy production.

Next was named as similar to retinoic acid-induced protein 1, which acts as a transcription regulating factor and is involved in development and differentiation of neural cells. The 7th protein was identified as an ubiquitin carboxyl-terminal hydrolase PGP9.5, which is known as an enzyme related to neuronal system and endocrine system. The peroxiredoxin was followed by and acts as an important antioxidant, protecting cells themselves by elimination of H_2O_2 produced from inside cells. The last one was beta-globin which is a part of hemoglobin that is well

known as a transporter of oxygen within erythrocyte.

The aspects of changes of all spots identified in this experiment are scrutinized. Interestingly the enzymes involved in the production of energy showed, despite the wide degree of differences, high activities in animals housed in LP (creatine kinase, isocitrate dehydrogenase, malate dehydrogenase, and triosephosphate isomerase. Table 2).

DISCUSSION

It is confirmed that the reproduction of golden hamsters used for present experiment is regulated by the photoperiod and the pineal gland mediates that. The photoperiodic informations are transmitted to the reproductive endocrine system, and SP induces inactive reproductive function. And the consequences of the regression are accompanied by numerous physiological changes, moreover it has been prominent that the proteins expressed in hypothalamus were modified upon the reproductive situations.

The metabolism might be resulted in anomalous consequences by the continuing stress on animals because this experiment took long period of time. Thus the changes of behavior and body weights were examined. From the beginning to the end of the experiment, no behavioral differences were observed in all animals without regard to presence and absence of the pineal gland. The body weights showed no significant alterations among animal groups, but there was an increasing tendency of body weights in animals housed in SP.

The results seem to suggest a sort of relationship between body weight and behavior. Golden hamsters breed for a restricted time period when they move around briskly in order to forage possible food and avoid from the potential predators for themselves and their offsprings, keeping the normal range of body weights. On the other hand, when they begin to contact the environment like winter climates, their moving activities also start to sluggish, leading to future torpor and eventually generating relatively an increase of body weights. This divergence could be reflected and imprinted in some part of their body upon the seasonal changes, as a biological clock or other forms.

The reproductive functions of golden hamsters were determined by photoperiod and pineal gland. The animals housed in LP with intact pineal gland persisted the active sexual activities but animals in SP showed completely regressed testes. This effect of SP was vanished in the pinealectomized animals, implying the involvement of pineal gland in regulation of reproduction. Blood melatonin levels were low at day time and significantly elevated at night in the animals with intact pineal gland regardless of photoperiod. This increase of melatonin at night was reduced into the levels of day times without respect to photoperiod in pinealectomized animals. Thus the pineal gland is thought to transmit the effects of photoperiod into reproductive endocrine system, and regulates sexual activities. Thus it is proper to conclude that reproductive functions are sustained in animals possessing low level of melatonin by removal of pineal gland.

The length of elevated levels of melatonin is correspondent to the length of time of darkness. As dark period of time is lengthened in animals housed in SP, the time period of melatonin released is prolonged in animals in SP compared to animals exposed to LP. Thus that elongated secretion of melatonin evokes reproductive regression. Therefore, if the pineal gland is eliminated from the body of animals, leading blood levels of melatonin to the levels of day time, ultimately reproductive activities are kept with the same result of the case of animals exposed to 24 hours of lighting.

All animals consequently ended up like dichotomy, that is, animals with reproductively active and inactive animals. The results were reflected by differing changes measured as physiological parameters. Histologically completely regressed testes showed thin walls of seminiferous tubules and no matured spermatozoa in it. The other animals demonstrated remarkably thick seminiferous tubules and plenty of matured spermatozoa in it. Testicular index also

showed the same results as analyzed by the absolute testicular weights.

The gonadotropins, FSH and LH, were significantly reduced in 5 weeks in the serum of animals housed in SP. However there were a tendency to increase in 10 weeks but no significance. These results are consistent with the previous reports in which the gonadotropins spontaneously begin to increase for the next breeding season, which is called refractory period (Tournier et al., 2009; Ogilvie & Stetson, 1990; Hong & Stetson, 1988). The refractory period is a procedure for the animals to gain spontaneously the reproductive functions during which the animals are insensitive to photoperiod. If the animals are subjected to keep in SP for long term, the animals obtain sexual activity in a proper time and once they gain it they do not reveal the original impacts of SP. In order for the animals in SP to respond in a gonadal regression, they should be put in LP for a suitable period of time. In natural environment, the reproductive activity would begin to involute following the autumnal equinox and to gain from the spring equinox. However the endocrine system of the animals at late winter has already been prepared for the next breeding season, demonstrating high levels of FSH, LH, and gonadal testosterone. In that case the animals get offsprings right away in a favorite ambient environment, keeping their species forever.

More interesting phenomena were seen in the expression of hypothalamic proteins detected on the 2d PAGE upon reproductive functions. The hypothalamic proteins from the LP animals were darker than those from SP animals. Those proteins are all involved in the production of energy, although the shadowing degree of the spots are widely various, in the process of glycolysis and Krebs cycle. These findings suggest that animals in LP are actively moving on the basis of more production of ATP for seeking food required and preventing themselves and their offsprings from the potential predators. These speculations indicate that the SP animals consider the SP as a nonbreeding season like winter, producing less amounts of energy, thus moving slow, resulting in gaining more body weights and preparing for the torpor.

The roles of the hypothalamic proteins identified are briefly summarized below. Aconitase, also called aconitate hydratase, is an enzyme converting citric acid into isocitric acid, is produced from the genes in cell nucleus, and transported into mitochondria where it is involved in the production of energy. It also protects DNA in mitochondria. There was no clear changes of this protein between LP and SP animals. The creatine kinase catalyzes the synthesis and the degradation of creatine phosphate, producing ATP in moving the bodily muscles. This enzyme is distributed high in skeletal muscle, cardiac muscle, smooth muscle, and brain, but are not present in other tissues and organs. Thus measurement of activity of this enzyme in blood lead to understand progressive muscle dystrophy, early diagnosis of heart attack, damages of organs, and changes of permeability of cell membrane. The activity of this protein is a little higher in males than females and augmented by exercise and intramuscular injection. As the LP animals move more actively than SP animals, these results have something to do with the known functions of high levels in LP animals. The isocitrate dehydrogenase 3(NAD^+) alpha catalyzes isocitrate into α-ketoglutarate and CO_2 in Krebs cycle while converting NAD^+ into NADH that produces ATP in the electron transport system. This enzyme was expressed much higher in hypothalamus of LP animals than in SP animals.

The cytosolic malate dehydrogenase catalyzes malate into oxaloacetate in Krebs cycle, resulting in NADH production (reversible reaction). And this enzyme is involved in the process of gluconeogenesis in which glucose is produced. The triosephosphate isomerase transforms reversibly dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate in glycolysis, essentially resulting in energy production. The deep deficiency of this enzyme develops outbreak of serious neurological disorder in human. The two enzyme described above were expressed much higher in hypothalamus of LP animals than in SP animals,

meaning that LP animals require even more energy than SP animals. In the light of reproduction that demands much energy, it is reasonable that LP animals should produce more energy than SP animals, despite of hypothalamus. The increased energy might be related to the GnRH synthesis and indirectly constant cell divisions in the course of spermatogenesis in testes.

The other proteins showed no plain differences between LP and SP animals. The retinoic acid-induced protein 1 acts as a transcription regulating factor and is involved in development and differentiation of neural cells. The ubiquitin carboxyl-terminal hydrolase PGP9.5 is known as an enzyme related to neuronal system and endocrine system, and is contained in a 1-2% of proteins of brain. The peroxiredoxin acts as an eminent antioxidant, and protects cells themselves by elimination of H_2O_2 produced from inside cells. This enzyme ranges widely from the prokaryote to eukaryotic cells and has been found until now six kinds in mammals. The last beta-globin is a part of hemoglobin that is well known as a transporter of oxygen within erythrocyte. Taken together, although hypothalamic specific proteins were not recognized, the proteins presented here and other proteins unidentified in the experiment might be enough to distinguish reproductive situations.

Recently a variety of attempts have been proceeding by using proteomics (Gharesi-Fard et al., 2010). In some cases reported at present, a diagnosis of diseases and effects of treatment (cerebral ischemic injury and cancer (Karpova et al., 2010; Sunget et al., 2009)), toxic influences (Shu et al., 2009), day-night variations in relation to circadian rhythms (Moller et al., 2007), the effects of steroids on pituitary gland (Blake et al., 2005), and the impact of microgravity (Sarkar et al., 2008), etc. are characterized. In addition to those mentioned above, the applicability of proteomics in a numerous tissues of animals is being under examination. Thus the proteomics integrates all the unnatural incidents as well as normal occurrences. Therefore, the results of the present study suggest the potential biomarkers collectively to be able to discern the fertility and infertility by approaching the proteins expressed in organs in relation to reproductive endocrine system, and further apply to the development of disease diagnosis and new drugs.

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