Serum testosterone assay

In study week 13 the mice were deeply anaesthetised with an i.p. injection of 4-mg Nembutal solution, and blood was collected by cardiac puncture to measure serum testosterone levels. An indirect competitive enzyme immunoassay (Cayman Chemicals, MI, USA) was carried out to measure serum testosterone levels in the vaccinated (Group 1) and control male mice (Group 2). The assay was carried out with diethyl ether extracted serum (according to the manufacturer's instructions) diluted 1:2 and 1:10 in immunoassay (EIA) buffer and used in duplicate on 96-well plates pre-coated with mouse monoclonal antibody to rabbit IgG. Competition was generated by simultaneously incubating the plate at room temperature for 2 h with 50 μl/well standard testosterone or 50 μl diluted sera, 50 μl/well acetylcholinesterase linked to testosterone (tracer) and 50 μl/well specific rabbit antiserum to testosterone. The wells were washed 5× with wash buffer and developed by incubating wells for 2 h with 200 μl/well Ellman's reagent (Cayman Chemicals). The plate was read at A_{405} and the serum concentration (average mean of 1:2 and 1:10 dilution) of testosterone was determined using a standard curve derived from known standards.

Epididymal sperm count

Following sacrifice the abdomen was opened, the left cauda epididymis excised, weighed and 10 mg epididymis was used to count total sperm numbers into a Neubauer counting chamber [16]. The right cauda epididymis was used to examine histological responses following immunisation.

Histological evaluation of reproductive organs

Following necropsy, the testes, epididymis, prostate, seminal vesicle, ovary and uterus from the vaccinated and control mice were weighed and fixed in 10% (v/v) buffered neutral formalin. Paraffin embedded 5 μm sections were stained with haematoxylin and eosin and Masson's trichome. In brief the Masson's trichome staining was carried out with the rehydrated sections on the slides by pre-treating for 30 min with 10% (v/v) trichloroacetic acid solution containing 1% (w/v) potassium dichromate and quickly rinsed in water followed by a nuclei staining with Weigerts iron haematoxylin for 10 min. Tissue sections were washed in running water for 10 min and 'blued' in lithium carbonate (reaction was confirmed under a light microscope). The connective tissue reactivity was visualised by treating the sections in 2% (v/v) orange green (in distilled water) for 8 min and rinsed 2× in 1% (v/v) acetic acid solution. The sections were stained in 1% (w/v) ponceau-acetic acid solution for 5 min and rinsed rapidly in water before differentiated in 1% (w/v) phosphomolybdic acid for 5–10 min or until collagen turned colorless. Sections were washed 2× with 1% (v/v) acetic acid solution, and counterstained with 0.4% (w/v) methyl blue for 2 min. This protocol was found to differentially stain gonadal germ cells and supporting tissues. Sections onto the slides were then dehydrated through 95% ethanol, absolute ethanol, cleared in xylene and mounted in DPX.

Quantitative analysis of the seminiferous epithelium of the testes (Table 1) was carried out to evaluate the level of spermatogenesis. In brief, using a 20× objective, 50 random tubular views per (three to five tubules/view) testicular section were examined and given a score. Quantitative analysis of the level of oogenesis in the vaccinated and control ovaries was conducted using criteria described in Table 2. With the 5× and 10× objectives ovarian sections were examined for specific scores and results were compared between vaccinated and control ovaries. The above examinations were carried out blind, by randomly coding the samples and not revealing the treatments until the end of the study. The histological examinations of epididymis, prostate and seminal vesicle were carried out at 10–20× objectives to evaluate differences between vaccinated and control organs.

Statistical analysis

Continuous data between control and experimental groups were compared with a Kruskal-Wallis unpaired χ^2-test using the Statistical Package for Social Sciences (SPSS) version 12.0 (SPSS, Chicago, IL, USA). A difference was considered significant at the $p < 0.05$ level. Data are reported as the mean ± S.E.M.

Table 1 A modified criteria [17] used to quantify level of spermatogenesis in the vaccinated and control testes stained with Masson's trichome

Scores	Criteria used
6	Complete spermatogenesis; characterised by regularly thickened germinal epithelium and a visible lumen; red sperm tail or blue sperm head coverage >50–80% in the lumen.
5	Regularly/irregularly thickened germinal epithelium with a visible lumen, <50% lumen coverage with dull colored sperm tail.
4	Disorganised germinal epithelium, marked sloughing off germ cells and obliteration of the lumen. Many spermatozoa/spermatids (>10) present
3	Only a few spermatozoa (<5–10) and/or spermatids (<10) present in the tubules. No visible lumen.
2	No spermatozoa and only a few spermatids (<5–10) present
1	No spermatozoa/spermatids but several or many spermatocytes present

Testicular score criteria 1–6 was used in this study to compare and evaluate the level of spermatogenesis in the vaccinated and control testes.

Table 2 Criteria designed to quantify level of oogenesis in ovaries following Masson's trichome staining

Scores	Criteria designed
6	Presence of a corpus luteum, a well circumscribed large collection of cellular bodies lacked oocyte (this does not make sense—but I donot know how to change it). The large steroidogenic luteal cells (granulosal) and small steroidogenic luteal cells (thecal) appeared as a closely packed cellular mass without antrum. The vascular network is well developed in the cellular mass.
5	Presence of graffian or antral follicles, contained an oocyte with a visible nucleolus, more than five layers of granulosa cells, and/or an antral space within the granulosa cell layers.
4	Regressing corpus luteum is characterised by small clusters of lightly stained cellular mass (goldner trichome, Fig. 4) lacked antram and oocyte. There is reduction in the number of large and small steroidogenic luteal cells and degeneration or loss of endothelial cells in the vascular network.
3	Secondary follicles (pre-antral) comprised an oocyte with a visible nucleolus surrounded by two to five layers of granulosa cells and lacked an antral space
2	Presence of primary follicles comprised an oocyte surrounded by a single layer of cuboidal granulosa cells.
1	Premodial follicles embedded in ovary, where oocytes are lined by a single layer of flattened epithelial cells.

Ovarian score criteria 1–6 was used to compare level of oogenesis in the vaccinated and control ovary.

Results

Vaccine designing and detection of fusion protein

In this study, we engineered a vaccine on a pcDNAV5-HisB plasmid (Fig. 1), where GnRH-I-T-helper epitopes were linked to the N terminus of a V5 epitope. The complete GnRH-I and T-helper encoding genes, the positions of the start and stop codons and the integrity of the open reading frames were determined by sequence analysis. The fusion protein open reading frame in pcDNAV5-HisB was found to contain a 533-bp long fragment, coding for 174 amino acids protein. To monitor the expression of the fusion protein in transfected COS1 cells and in culture supernatant, the blotting membrane was labelled with an anti-V5 antibody. Western blotting analysis revealed that both the transfected COS1 cells and culture supernatant contain 18.871 kDa fusion protein (data not shown) but did not develop this molecular mass in the transfected rat muscle. The expression of fusion protein in the transfected rat muscle was, therefore, detected using immunohistochemistry. Transfected rat muscle displayed fusion protein, distributed throughout the cells, which was absent in the control sections (Fig. 2).

Gross physiological effect of immunisation

All the mice tolerated the intramuscular injections of plasmid DNA and did not suffer from agitation, fever or inflammatory swelling at the injection sites. There was no apparent discomfort exhibited by any of the mice, either on recovery from anaesthesia after the injection or at any point during the course of the experiment. There was no visible change in food consumption among the groups of mice used in this study. None of the mice injected with plasmid DNA showed signs of morbidity or mortality during this study. Post-mortem inspection of the treated mice particularly at injection sites did not reveal any inflammatory reaction. There was no significant difference in body weight between the vaccinated and control males in study weeks 0 (22.80 ± 0.97 g and 23.02 ± 1.23 g) and 12 (54.56 ± 0.77 g and 52.02 ± 1.37 g), respectively. Similarly, vaccinated and control female mice showed no significant difference in body weight in study weeks 0 (19.40 ± 1.15 g and 20.07 ± 1.60 g) and 12 (41.90 ± 1.35 g and 43.12 ± 0.05 g), respectively. At necropsy significant reduction of the weight of the combined prostate and seminal vesicles in the vaccinated males (356 ± 46.15 mg, $p = 0.015$) were observed compared to control prostate and seminal vesicles (452 ± 38.99 mg). A significant reduction of the weight of testis was observed in the vaccinated testes (129 ± 9 mg, $p = 0.20$) compared to the control testes (139 ± 11.00 mg). Ovaries obtained from the vaccinated females also showed a significant (18 ± 2 mg, $p = 0.004$) reduction in weight in contrast to the control ovaries (22 ± 2 mg).

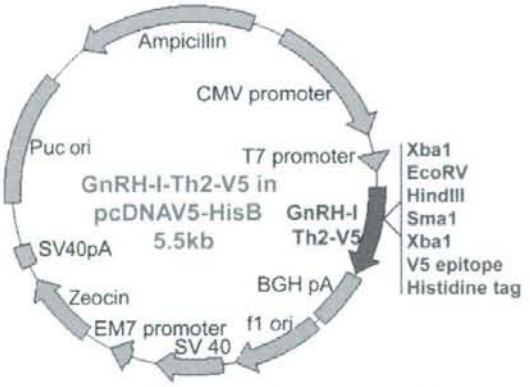

Figure 1 The 5.5 kb long pcDNAV5-HisB plasmid map indicating the relative positions of the vaccine construct (blue color, GnRH-I), the immediate early to BGH polyadenylation signal (BGHpA) and down to T7 promoter sites. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

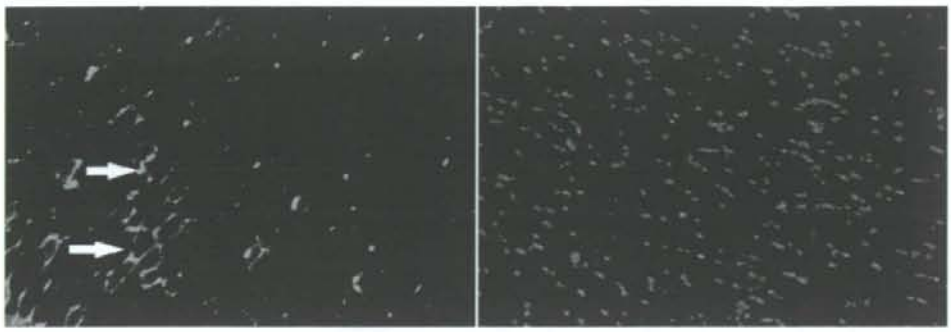

Figure 2 Fluorescence detection of fusion protein (white arrow) in the rat anterior quadriceps muscle transfected (left) with the vaccine construct in pcDNAV5-HisB plasmid. Transfected and control (right) muscle sections were labelled with mouse monoclonal anti-V5 antibody, FITC labelled goat anti-mouse IgG1 and bisbenzimide trihydrochloride. Transfected muscle showed V5 antigen-specific green fluorescent (arrow) reactivity (left) dispersed through out the muscle fibers, which was absent in the control section (right), magnification 10×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Anti-GnRH-I antibody response

Immunisation resulted in the development of GnRH-I specific IgG1 antibody response in vaccinated male (Group 1) and female mice (Group 3) (not shown graphically). The vaccinated female animals showed an early (week 3) and higher immune response compared to the vaccinated male mice, in which the response was detected in study week 6. Mice 2 and 4 in Group 3 had endpoint titres, which were consistently higher (end point dilution titre reaching a maximum of 1/25,600 in study weeks 9 and 12 compared with 1/3200 in untreated plasma). By the end of the study, the vaccinated males (Group 1) showed significant antibody titres; 1/12,800 in one mouse, compared with the rest at 1/6400 in study weeks 9 and 12.

Serum testosterone level

The volume of diluted serum used for EIA (enzyme immunoassay) was 50 μl/mice. The test serum was purified using diethyl ether and diluted in Tris–acetate EDTA (TAE) buffer to ignore non-specific reaction of the reactive proteins normally present in sera. All the samples were run at the same time to avoid inter-assay variation. Significant reduction of the mean serum testosterone concentration (pg/ml) ± S.E.M. was found in vaccinated male mice (Group 1, 1188.16 ± 462.93, $p = 0.021$) compared with the Group 2 controls of 3359.25 ± 170.52. The highest reduction of serum testosterone concentrations were seen in mouse 1, 4 and 5 (Group 1, Fig. 3a).

Epididymal sperm analysis

Morphological evaluation of the sperm obtained from the tail of epididymis (Groups 1 and 2 mice) did not reveal major defects in their head and tail pieces. Sperm obtained from the epididymis of Group 1 mice (Fig. 3b) showed a significant reduction in counts ($1.54 \pm 0.69 \times 10^6$, $p = 0.014$) compared to Group 2 control mice ($2.54 \pm 0.33 \times 10^6$).

In vivo fertility analysis

Following the first (13 ± 2.00, Fig. 4a) and second (10.60 ± 2.68, Fig. 4b) matings, a slight reduction of implants was seen in the virgin females mated with vaccinated (Group 1) and control males (Group 2, 16.40 ± 0.79, Fig. 4a and b). The vaccinated female mice (Group 3) mated

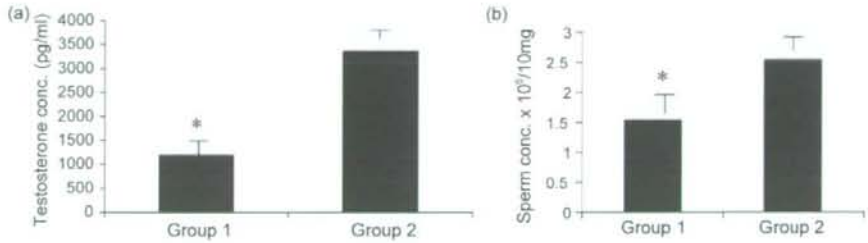

Figure 3 (a) Results of competitive EIA showed significant reduction of serum testosterone levels in the vaccinated male mice (Group 1) compared to naked pcDNAV5-HisB treatment (Group 2). *$p = 0.01$. (b) Results of epididymal sperm count showed significant reduction of sperm concentrations in vaccinated (Group 1) male mice ($1.54 \pm 0.69 \times 10^6$) compared to naked pcDNAV5-HisB treatment (Group 2, $2.54 \pm 0.33 \times 10^6$). *$p = 0.014$.

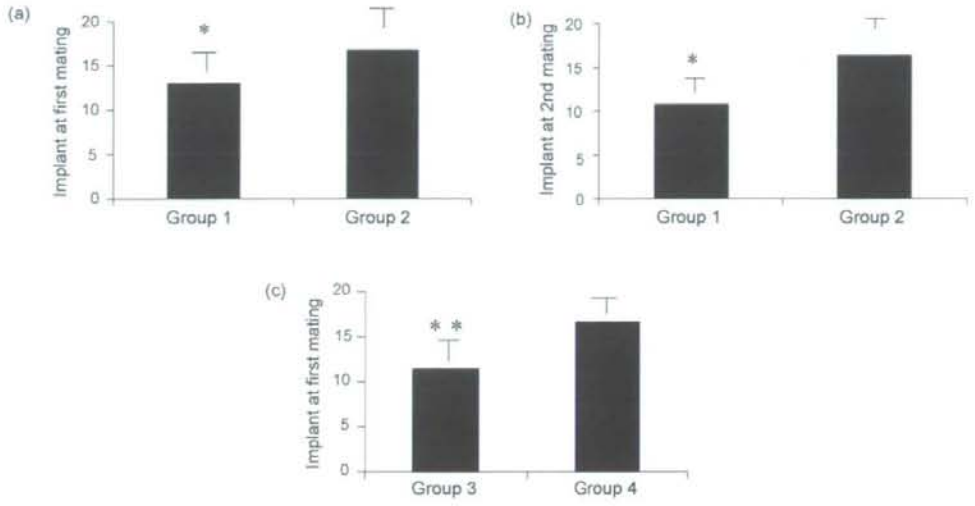

Figure 4 *In vivo* fertility assay of first (study week 10) and second (study week 12) mating trials between vaccinated (Group 1) male and untreated female mice showed significant reduction of implants in the first ((a) 13 ± 2.00) and second mating ((b) 10.8 ± 1.68). The first (study week 12) mating trial between vaccinated female (Group 3) and untreated control male mice showed implants ((c) 11.4 ± 1.11) which was much lower compared to the implants (16.6 ± 0.85) obtained from Group 2 and 4 mice mated with untreated female and male mice, respectively. $^{*}p < 0.05$ and $^{**}p < 0.005$.

with untreated males in study week 12 showed significantly lower implants (11.4 ± 1.82, $p = 0.004$) compared to Group 4 controls (16.6 ± 1.14, Fig. 4c).

Microscopic evaluation of testes and ovary

Results of microscopic evaluation showed a significant reduction (score 6, $p = 0.003$) in spermatogenesis in the seminiferous tubules of vaccinated mice (Table 3). There were no significant differences of scores 4 and 3 between vaccinated and control mice. However, overall the tissue changes produced in the vaccinated tubules comprised a lack of sufficient sperm and low densities of spermatogenic cells in the seminiferous tubules (Fig. 5a). The interstitial cells obtained from mice 1, 4 and 5 in Group 1 showed cytoplasmic atrophy compared to control sections (Fig. 5b). Epididymis obtained from the vaccinated mice showed low sperm concentrations in the head and tail. A low density of cilia embedded in the lining epithelium of epididymis was also observed. There was reduction in the concentration of secretory granules in the lining epithelium of the prostate. Seminal vesicles obtained from vaccinated mice showed a reduction in stored secretions compared to the secretion rich alveoli in control seminal vesicles.

Histological evaluation of vaccinated ovaries showed no significant differences in the appearance of the primordial ($p = 0.102$), primary ($p = 1.000$) and pre-antral follicles ($p = 0.625$) compared to control ovaries. The numbers of fully mature corpus luteum (CL) were subnormal ($p = 0.306$) in the vaccinated mice (Fig. 5c). However, overall the numbers of regressing corpus luteum (RCL) were much lower ($p = 0.0001$) in the vaccinated ovaries (Fig. 5c) than in the control mice (Fig. 5d). A sharp decrease in the appearance of antral follicles in the vaccinated ovaries ($p = 0.013$, Table 3) indicated a state of impaired folliculogenesis following vaccination. Vaccination was found to increase overall stromal reaction and reduce vascular engorgement in the ovary.

Table 3 Results of suppressed gametogenesis in vaccinated male and female gonads

Scores	Group average testicular score ± S.E.M.			Group average ovarian score ± S.E.M.		
	Vaccinated (Group 1)	Control (Group 2)	*p*-Value	Vaccinated (Group 3)	Control (Group 4)	*p*-Value
6	14.2 ± 1.64	23.2 ± 2.39	0.003	6.4 ± 1.52	8.0 ± 2.7	0.306
5	29.4 ± 2.19	21.0 ± 1.39	0.012	19 ± 4.84	31.8 ± 6.7	0.013
4	5.4 ± 1.14	5.0 ± 1.58	0.374	7.4 ± 2.28	28.4 ± 4.9	0.0001
3	0.8 ± 0.45	0.8 ± 0.45	1.000	20 ± 4.41	22 ± 2.2	0.625
2	0.5 ± 0.45	–	–	9.8 ± 2.77	9.6 ± 1.5	1.000
1	–	–	–	84.8 ± 8.0	93.2 ± 5.6	0.102

Microscopic examination of seminiferous tubules of testes and ovarian follicles/corpus luteum from both vaccinated and control mice were evaluated by assessing morphology (Tables 1 and 2) and results were evaluated by a paired sample *t*-test to unveil the difference.

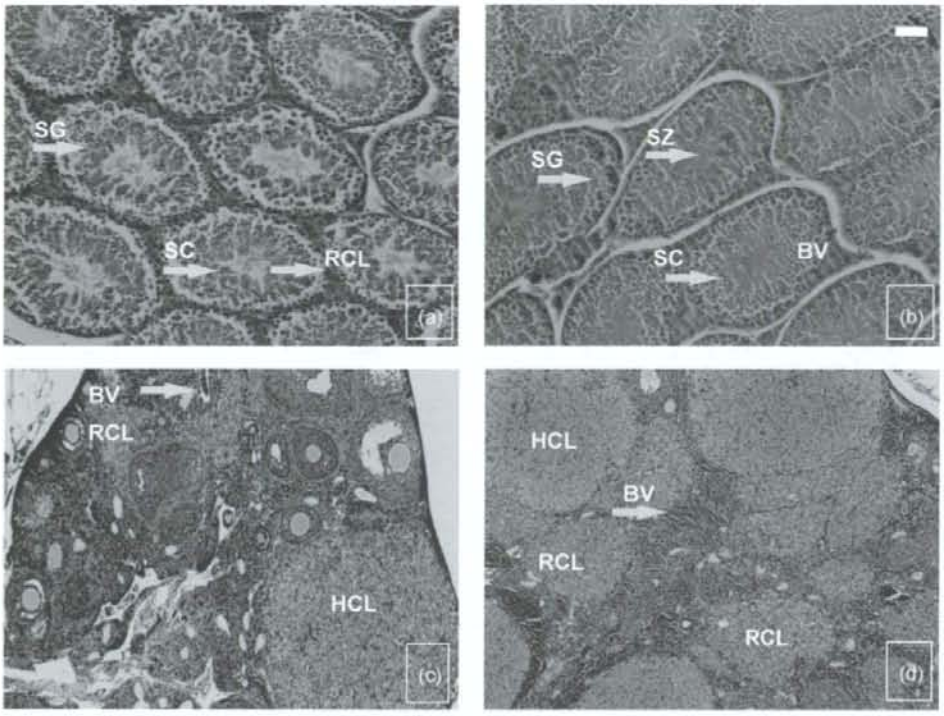

Figure 5 Microscopic evaluation of gonadal architecture in the vaccinated and control mice. Marked suppression of spermatogenesis was seen in the vaccinated testes (a) characterised by the lack of spermatozoa (SZ), reduce concentration of spermatogonium (SG) and spermatocytes (SC) compared to control tubular section (b) rich in spermatozoa, spermatogonium and spermatocytes. The vaccinated ovary (c) showed the lowest count ($p = 0.0001$) of regressing corpus luteum (RCL) and a reduction of healthy corpus luteum (HCL) compared to control sections (d). The HCL in the vaccinated ovary appeared larger than HCL in control section. The engorged blood vessels (BV) that were abundant in control ovary appeared few and tiny in the vaccinated ovary. Vaccination was found to increase overall stromal reaction in ovarian parenchyma. Magnification 5×.

Discussion

At present, studies are focused onto the immunogenic control of fertility axis and there is a significant amount of literature describing various targets for the development of contraceptive vaccines. These broadly fall into three categories: (i) targets inhibiting gamete production (GnRH-I, FSH, and LH), (ii) gamete function (zona pellucida proteins and sperm antigens), or (iii) gamete outcome (human chorionic gonadotrophin). Advantages of GnRH-I-based targets such as GnRH-I peptide conjugates [3,17] or GnRH-I fusion proteins [9,14] in animals have two beneficial goals, disruption of fertility and suppression of breeding behaviour [5,7,18,19]. One advantage of a GnRH-I immunoneutralisation strategy using genetic vaccines is that they are effective in several species, they are easy to construct, are viable at wide ranges of temperature and can be used for both males and females [7,16,18]. Genetic vaccines offer broad efficacy particularly for their ability to generate both cellular and humoral immune responses and the potential for world-wide uses even in low-resource settings [20]. Previous designs of vaccines used large proteins such as diphtheria toxoid (DT) or tetanus toxoid as a carrier in the semisynthetic GnRH-I vaccines [2,11], which were then subsequently replaced by T-helper epitopes interspersed in multiple GnRH-I units [14,16]. This was done to avoid carrier (DT/TT) induced epitope suppression [15] and also to communicate through an array of these T-cell determinants with major histocompatibility complex (MHC) across the spectrum in a polygenetic population. The antibodies generated are directed at GnRH-I, the bulk of the antibodies formed against the carriers (DT/TT) was avoided, and as expected no antibodies are formed against T non-B cell peptides used as carriers [21,22].

However, genetic immunisation studies revealed that the DNA vaccine without a vector system can be relatively inefficient [16], as most of the DNA is rapidly internalised and cleared by macrophages [21] and repeated administration of plasmid DNA is required to increase the duration and level of transgene expression [23]. The low potency of genetic immunisation in large animals is that the injection of microgram doses of DNA results in the translation of only nanogram doses of protein [24] and most of the encoded proteins are not always secreted from the translation sites to stimulate innate immune system [18]. Current

doses of DNA needed in humans and larger animals are in the order of milligram amounts and ways to reduce this dose are required to keep the manufacturing costs at a reasonable level. Indeed, all these efforts are synergistic and require engineering for the improved antigen expression following DNA delivery *in vivo*. In this study, we designed and engineered a plasmid DNA vaccine encoding GnRH-I and T-helper epitopes. The T-helper epitopes were engineered into the genome sequence to augment a humoral immune response. A GnRH-I leader sequence at the N-terminal and a V5 epitope (neutralizing region of the feline immunodeficiency virus) at the C-terminal of the vaccine were incorporated in order to enhance the releasing property of the fusion protein and to induce sufficient anti-GnRH-I antibody responses to cause infertility.

Male and female mice were immunised with the vaccine in saline solution in order to develop a DNA vaccine without vector incorporation and sustained release of translated protein was maintained using multiple vaccine delivery. The intramuscular injection of plasmid DNA vaccine in mice was found to induce an antigen-specific IgG antibody response. The antibody response kinetics appears slightly delayed after three immunisations in saline-mediated delivery compared to an early response in viral vector mediated immunisation [16]. A difference in antibody kinetics through particle mediated epidermal delivery of influenza DNA vaccine has also been observed and greatest antibody responses were detected at the last time point tested (day 56) [25]. The immune response to the DNA vaccine may be qualitatively different than the response to fusion protein [14] or peptide conjugates [17,26]. Mice immunised with GnRH-I-TT conjugate [17] in study weeks 0, 2, 4 and 6 showed GnRH-I specific IgG responses in study week 4 (A_{450} is 0.08 ± 0.05) and peaked in study week 6 (2.00 ± 0.03). Genetic immunisation with a hepatitis B vaccine studies suggest that two doses of vaccine are required to elicit antibody responses in naïve subjects, and three doses of vaccine are required for maximal response [27]. This study provides evidence that three and four doses of vaccines for females and males, respectively, are required for generating humoral immune response. A particle mediated epidermal delivery with hepatitis B DNA vaccine trial demonstrated that three doses of DNA immunisation is required to effectively induce humoral responses in 100% of the immunised subjects at levels that exceed the minimum required for protective immunity in humans [20]. A plasmid DNA vaccine without adjuvant or vector system appeared to induce low levels of cellular delivery of plasmid DNA and insufficient stimulation of the immune system [18]. Indeed, we have improved a few aspects of DNA vaccination by utilizing the backbone of pcDNAV5-HisB, using a leader sequence and incorporation of T-helper epitopes; our approaches have addressed the relative contributions of these alterations to vaccine potency as the translated protein is released in the extracellular environment.

Immunisation of male mice with this vaccine reduced serum testosterone concentration, impaired testicular spermatogenesis and suppressed fertility *in vivo*. Female mice were more responsive to the vaccine than males in terms of anti-GnRH-I antibody responses and reduced implant numbers. Evaluation of folliculogenesis in the immunised females showed no significant differences between the primordial and primary ovarian follicles. This finding indicates that follicular recruitment, which is a spontaneous process in mice, is not altered following immunisation. Once the follicles are recruited at primordial follicle stage, FSH controls follicle differentiation and growth. Progressive development of ovarian follicles is, therefore, controlled by pituitary gonadotrophins, which is regulated by hypothalamic GnRH-I [28]. The progressive development of ovarian follicles in vaccinated mice from pre-antral to antral stage was arrested sharply as there was significant reduction ($p = 0.013$) of antral follicles in the vaccinated ovary. Significantly, the lowest number of regressing corpus luteum ($p = 0.0001$) was noted in the vaccinated mice compared to the controls indicating that the vaccinated mice had a LH surge, which is insufficient to cause ovulation and formation of corpus luteum (CL, Fig. 4). The reduced number of antral follicles and mature CL in the vaccinated ovary indicated an overall suppression of pituitary gonadotrophins (FSH and LH) following immunisation. A dense stromal tissue reaction interspersed around remaining oocytes, follicles and CL in the vaccinated ovary could be due to depletion of follicles and CL following vaccination. The vaccinated and control female mice were allowed to become pregnant (10–14 days) before sacrifice in order to bring uniformity in ovarian structure and function, without which it would have been difficult to evaluate responses of immunisation at different stages of oestrous. However, these results indicated that the antibody-mediated neutralisation of GnRH-I was not complete, especially since gametogenesis and steroidogenesis were not completely suspended.

The present study provides evidence that plasmid DNA vaccine in saline solution is efficient at inducing GnRH-I specific IgG immune response in mice. A viral vector system [16] or particle-mediated epidermal delivery of plasmid DNA vaccine [25] on the other hand delivers earlier and higher immune responses. A priming and first booster of the vaccine in a suitable vector or carrier system and subsequent immunisation in saline solution could be a potential approach to enunciate applicability of a plasmid DNA vaccine. However, the results of multiple antigen delivery in saline solution appears promising with respect to anti-GnRH-I antibody responses, reduced serum testosterone levels, suppressed gametogenesis and reduced fertility *in vivo*. The reduction of testosterone secretion indicates a potential use of the GnRH-I-T-helper vaccine in farm animals where androgen neutralisation is required especially to control spaying, or to eliminate boar taint [29,30]. Our results clearly demonstrate that the plasmid DNA vaccine we have designed, containing signal(s) to be secreted, elicited an anti-GnRH-I antibody response in the absence of a carrier and adjuvant to enable fertility to be disrupted. The immunogenecity of this vaccine could be enhanced by designing a vaccine containing more repeats of GnRH-I, framework engineering to help the translated protein to be secreted *in vivo* and priming/boosting with a GnRH-I-T-helper epitope fusion protein. Furthermore, our previous experience has shown that there is species variation in response to GnRH and indeed DNA vaccination [2], therefore, studies are on-going to demonstrate applicability in the field and the potential of this vaccine which has shown potential in laboratory models.

Acknowledgments

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References

[1] Stanislaus D, Pinter JH, Janovick JA, Conn PM. Mechanisms mediating multiple physiological responses to gonadotropin-releasing hormone. Mol Cell Endocrinol 1998;144:1–10.

[2] Ferro VA, Khan MAH, Colston A, Aughey E, Mullen AB, Waterston MM, et al. Efficacy of an anti-fertility vaccine based on mammalian gonadotrophin releasing hormone (GnRH-I)–-a histological comparison in male animals. Vet Immunol Immunopathol 2004;101(1/2):73–86.

[3] Earl ER, Waterston MM, Aughey E, Harvey MJA, Matschke C, Colston A, et al. Evaluation of two GnRH-I based vaccine formulations on the testes function of entire suffolk cross ram lambs. Vaccine 2006;24(16):3172–83.

[4] Emons G, Grundker C, Gunthert AR, Westphalen S, Kavanagh J, Verschraegen C. GnRH antagonists in the treatment of gynecological and breast cancers. Endocr Relat Cancer 2003;10:291–9.

[5] Ferro VA, Stimson WH. Immunoneutralisation of gonadotrophin releasing hormone: a potential treatment for oestrogen-dependent breast cancer. Eur J Cancer 1997;33:1468–78.

[6] Jinshu X, Jingjing L, Duan P, Zheng Z, Ding M, Jie W, et al. A synthetic gonadotropin-releasing hormone (GnRH) vaccine for control of fertility and hormone dependent diseases without any adjuvant. Vaccine 2005;23(40):4834–43.

[7] Talwar GP, Raina K, Gupta JC, Ray R, Wadhwa S, Ali MM. A recombinant luteinising-hormone-releasing-hormone immunogen bioeffective in causing prostatic atrophy. Vaccine 2004;22(27/28):3713–21.

[8] Godfrey SI, Walkden-Brown SW, Martin GB, Speijers EJ. Immunisation of goat bucks against GnRH to prevent seasonal reproductive and agonistic behaviour. Anim Reprod Sci 1996;44(1):41–54.

[9] Jinshu X, Jingjing L, Duan P, Zheng Z, Ding M, Jie W, et al. The immunogenecity of recombinant and dimeric gonadotrophin-releasing hormone vaccines incorporating a T-helper epitope and GnRH or repeated GnRH units. J Immunol Method 2004;289:111–22.

[10] Killian G, Miller L, Rhyan J, Doten H. Immunocontraception of Florida feral swine with a single-dose GnRH vaccine. AJRI 2006;55(5):378–84.

[11] Ferro VA. Current advances in antifertility vaccines for fertility control and noncontraceptive applications. Expert Rev Vaccine 2002;1(4):443–52.

[12] Ferro VA, Mordini E. Peptide vaccines in immunocontraception. Curr Opin Mol Ther 2004;6(1):83–9.

[13] Hsu CT, Ting CY, Ting CJ, Chen TY, Lin CP, Whang-Peng J, et al. Vaccination against gonadotropin-releasing hormone (GnRH) using toxin receptor-binding domain-conjugated GnRH repeats. Cancer Res 2000;60:3701–5.

[14] Gupta JC, Raina K, Talwar GP, Verma R, Khanna N. Engineering, cloning, and expression of genes encoding the multimeric luteinizing-hormone-releasing hormone linked to T cell determinants in *Escherichia coli*. Prot Expr Purif 2004;37(1):1–7.

[15] Sad S, Chauhan VS, Arunan K, Raghupathy R. Synthetic gonadotrophin-releasing hormone (GnRH) vaccines incorporating GnRH and synthetic T-helper epitopes. Vaccine 1993;11:1145–50.

[16] Khan MAH, Ferro VA, Koyama S, Kinugasa Y, Song M, Ogita K, et al. Immunisation of male mice with a plasmid DNA vaccine encoding gonadotrophin releasing hormone (GnRH-I) and T-helper epitopes suppresses fertility in vivo. Vaccine 2007;25:3544–53.

[17] Khan MAH, Prevost M, Waterston MM, Harvey MJA, Ferro VA. Effect of immunisation against gonadotrophin releasing hormone isoforms (mammalian GnRH-I, chicken GnRH-II and lamprey GnRH-III) on murine spermatogenesis. Vaccine 2007;25:2051–63.

[18] Ulmer JB, Wahren B, Liu MA. Gene-based vaccines: recent technical and clinical advances. Trends Mol Med 2006;12(5):216–22.

[19] Levya JK, Millerb LA, Cynda Crawforda P, Ritcheyc JW, Rossa MK, Kathleen A, et al. GnRH mmunocontraception of male cats. Theriogenol 2004;62:1116–30.

[20] Fuller DH, Loudon P, Schmaljohn C. Preclinical and clinical progress of particle-mediated DNA vaccines for infectious diseases. Methods 2006;40(1):86–97.

[21] Fernando A, Rodriguez JR, Garzon A, Rodriguez D, Esteban M. Improving recombinant MVA immune responses: potentiation of the immune responses to HIV-1 with MVA and DNA vectors expressing Env and the cytokines IL-12 and IFN-gamma. Virus Res 2006;116(1–2):11–20.

[22] Finstad CL, Wang CY, Kowalski J, Zhang M, Li ML, Li XM, et al. Synthetic luteinizing hormone releasing hormone (LHRH) vaccine for effective androgen deprivation and its application to prostate cancer immunotherapy. Vaccine 2004;22:1300–13.

[23] Jang JH, Shea LD. Intramuscular delivery of DNA releasing microspheres: icrosphere properties and transgene expression. J Control Release 2006;112(1):120–8.

[24] Huygen K. Plasmid DNA vaccination. Microb Infect 2005;7(5/6):932–8.

[25] Drape RJ, Macklin MD, Barr LJ, Jones S, Haynes JR, Dean HJ. Epidermal DNA vaccine for influenza is immunogenic in humans. Vaccine 2006;24(21):4475–81.

[26] Ferro VA, Khan MAH, Latimer VS, Brown D, Urbanski HF, Stimson WH Immunoneutralisation of GnRH-I, without cross-reactivity to GnRH-II, in the development of a highly specific anti-fertility vaccine for clinical and veterinary use. J Reprod Immunol 2001;51:109–29.

[27] Rottinghaus ST, Poland GA, Jacobson RM, Barr LJ, Roy MJ. Hepatitis B DNA vaccine induces protective antibody responses in human non-responders to conventional vaccination. Vaccine 2003;21(31):4604–8.

[28] Jonathan SB. Reproductive physiology. In: Williams L, Wilkinsons, editors. Novak's Gynecology. 13th ed. Tokyo: L Williams and Wilkins; 2002. p. 149–72.

[29] Adams TE. Using gonadotrophin-releasing hormone (GnRH) and GnRH analogs to modulate testis function and enhance the productivity of domestic animals. Anim Reprod Sci 2005;88(1/2):127–39.

[30] Thompson DL. Immunisation against GnRH in male species: comparative aspects. Anim Reprod Sci 2000;60/61:459–69.

Association between serotonin transporter gene polymorphisms and depressed mood caused by job stress in Japanese workers

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Abstract. To estimate the genetic factors influencing depressed mood caused by job stress, a total of 243 employees at a manufacturing company and a local hospital in Japan (mean age 40.8±10.3 years) were recruited with informed consent. The Brief Job Stress Questionnaire was used to assess the present status of stress. Alcohol consumption and smoking were assessed as lifestyle factors. DNA samples were prepared to detect gene polymorphisms of serotonin transporter (5HTT), aldehyde dehydrogenase 2, D2 dopamine receptor, and cytochrome p450 2A6. The relationship between job stress, lifestyle factors and these polymorphisms was assessed for each gender. The level of depressed mood for female subjects was significantly higher among the carriers of two short (s/s) alleles of the 5HTT regulatory region compared with the carriers of one (s/l) or two (l/l) long alleles (Mann-Whitney U test, $p<0.05$). The odds ratio of depressed mood also confirmed this relationship for the female subjects, whereas there was no relationship for the male subjects. When social support was taken into consideration, the depressed mood score for those who had high support was significantly lower than for those who had low support, irrespective of 5HTT polymorphisms and gender. Job stress may elicit biological responses that contribute to depressed mood in relation to 5HTT polymorphisms, and social support may reduce depressed mood irrespective of 5HTT polymorphisms.

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Key words: serotonin transporter polymorphism, depressed mood, aldehyde dehydrogenase 2 polymorphism, D2 dopamine receptor polymorphism, cytochrome p450 2A6 polymorphism

Introduction

Genetic factors have been implicated in many lifestyle-related diseases such as cancer, cardiovascular disease, and osteoporosis (1-3). Genetic factors such as gene polymorphisms have also been linked with major depressive disorder, alcoholism, and nicotine dependence (4-7). Certain serotonin transporter (5HTT) gene polymorphisms have been associated with several dimensions of neurosis and psychopathology, especially anxiety traits (8). The acquisition of personal habits such as smoking and drinking has also been associated with gene polymorphisms; specifically, D2 dopamine receptor (DRD2) and cytochrome p450 2A6 (CYP2A6) polymorphisms have been linked to smoking (6,7) and aldehyde dehydrogenase 2 (ALDH2) polymorphisms to drinking (9).

On the other hand, the number of workers who suffer from job stress is increasing in Japan because of a prolonged recession, the increasing number of elderly employees, and the structural reorganization of companies (10). Increasing job stress may cause stress-related diseases and disorders such as coronary heart disease, hypertension, depression, insomnia, and substance abuse (11-14).

In order to avoid an increase in the incidence of stress-related diseases, it is necessary to measure the present level of job stress. Many questionnaires have been developed to assess job stress, e.g. the Job Content Questionnaire (JCQ) (15), WHO MONICA Psychosocial Optional Study Questionnaire (16), and the Brief Job Stress Questionnaire (17). The Brief Job Stress Questionnaire is particularly useful: consisting of only 57 questions it is easy to administer in the workplace, and being based on the JCQ and the MONICA questionnaire, it is reliable and valid. However, stress-related diseases are also lifestyle-related diseases that might result from complex interactions between genetic and environmental factors. Thus, in addition to stress estimation, the assessment of personal habits such as smoking and drinking is required as well. Job stress itself may also be involved in the development of detrimental lifestyle factors.

In the present study, the relationship between lifestyle and job stress estimated using the Brief Job Stress Questionnaire was analyzed referring to polymorphisms of the 5HTT, ALDH2, DRD2, and CYP2A6 genes.

Subjects and methods

Subjects. A total of 304 staff members at a local manufacturing company and a local hospital in the western part of Japan were recruited with informed consent. Since 61 of the employees declined to participate in the study, a total of 243 subjects was selected, of which 138 were male. We obtained written informed consent from all subjects. This study was approved by the Medical Ethics Committee of Kawasaki Medical School and Kawasaki Medical School Hospital (no. 52).

Lifestyle factors and measurement of job stress. Drinking and smoking were assessed as lifestyle factors. Alcohol consumption was evaluated by a self-assessment questionnaire and was expressed as grams of ethanol consumed per week. Smoking was assessed using the Brinkman index, which is determined as the number of cigarettes per day multiplied by the number of years since starting to smoke (18).

Job stress was calculated using the Brief Job Stress Questionnaire (17), which consisted of 57 items. Using this questionnaire, stressors such as work overload and personal relations, psychosomatic responses to stress, and social support can be assessed as well as depressed mood. While many parameters can be assessed, depressed mood was used as the stress reaction in the present study.

Measurement of polymorphisms. Blood samples were obtained at an annual health examination, and genomic DNA was extracted from leukocytes. To identify polymorphisms of the serotonin transporter (5HTT), aldehyde dehydrogenase 2 (ALDH2), D2 dopamine receptor (DRD2), and cytochrome P450 2A6 (CYP2A6) genes, their polymorphic loci were amplified using polymerase chain reaction (PCR).

The promoter activity of 5HTT is modified by sequence elements within the proximal 5' regulatory region. A 20-23 base pair repeat motif within this region occurs as 2 prevalent alleles, one consisting of 14 repeats (the short allele 's') and another of 16 repeats (the long allele 'l'). Fig. 1A shows the 5HTT polymorphism types. This polymorphic region has functional significance: 'l/l' homozygote lymphoblast cells produce 1.4-1.7 times the concentration of 5HTT mRNA than 's/l' and 's/s' cells, uptake of labeled serotonin in 'l/l' homozygote cells is two times greater than in 's/l' or 's/s' cells, and the protein produced from 'l/l' cells binds 30-40% more serotonin than that of cells with the short variant (19). The primers used for 5HTT were: forward, ATGCCAGCAC CTAACCCCTAATGT and reverse, GGACCGCAAGGTG GGCGGGA (19).

Individual drinking behavior is regulated not only by the social and economic environment but also by ethanol-metabolizing capacity. ALDH2 with a single-point mutation in exon 12 and atypical homozygotes (*2/*2) or heterozygotes (*2/*1) showed marked increase in blood acetaldehyde level compared with typical homozygotes (*1/*1) after alcohol consumption (5). After amplification using a set of primers (forward, CAAATTACAGGGTCAACTGCT and reverse, CCACACTCACAGTTTTCTCTT), the products were digested with MboII: *2 was digested but not *1. Fig. 1B shows the ALDH2 polymorphism types.

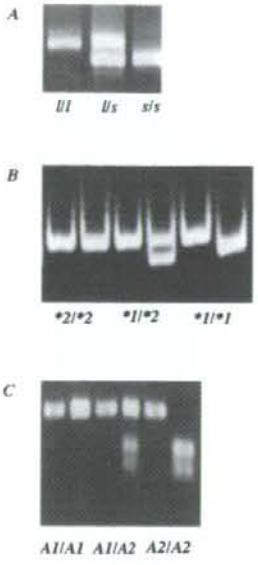

Figure 1. Representative types of polymorphisms in the 5HTT (A), ALDH2 (B) and DRD2 (C) genes.

Dopaminergic neurotransmission in the mesolimbic system is a recognized and critical target of many drugs of abuse, including alcohol, cocaine, and nicotine (20,21). Smokers carrying the A1 allele (TaqI indigestible) in the 3' flanking region of DRD2 receptor gene quit less frequently than those with the homozygous A2 allele (TaqI digestible) (22). Primers used for DRD2 were: forward, CCGTCGACG GCTGGCCAAGTTGTCTA and reverse, CCGTCGACCCT TCCTGAGTGTCATCA for DRD2 (23). Fig. 1C shows the DRD2 polymorphism types.

Variable CYP2A6 polymorphisms are classified into three groups by activity: normal inactivator (n), without copies of CYP2A6*2, CYP2A6*4, CYP2A6*6, and CYP2A6*12; intermediate inactivator (i), with heterozygosity for CYP2A6*9 or CYP2A6*12 and ~75% of wild-type n activity; and slow inactivator (s), with one or more copies of CYP2A6*2 or CYP2A6*4, or homozygosity for CYP2A6*9 or CYP2A6*12, and with ≤50% of n activity. All CYP2A6 polymorphisms were determined by sequence analysis according to the method of Schoedel *et al* (24).

Statistical analyses. The Mann-Whitney U test and the Kruskal-Wallis test were performed as non-parametric tests to compare differences in depressed mood, alcohol consumption, and the Brinkman index, for each polymorphism. Factor analysis was performed in order to explore the relationship among job stress, polymorphisms, and lifestyle factors. Logistic regression analysis was also performed to determine the risk of increment of depressed mood between each polymorphism and lifestyle factors. Odds ratios as estimated risk with 95% confidence intervals

Table I. Characteristics of subjects.

	Total (n=243)	Male (n=138)	Female (n=105)
Mean age	40.8±10.3	41.2±9.8	40.2±10.9
Drinkers			
Number	182	109	73
Rate	74.9	79.0	69.5
Average consumption (g/week)	124.9±179.4	220.8±205.4	84.1±126.0[a]
Smokers			
Number	112	93	19[a]
Rate	46.1	67.4	18.1
Brinkman index	462.2±285.6	503.8±282.5	258.4±205.6[a]
Depressed mood score	111.1±48.5	114.7±54.7	106.4±38.6
Stressors	25.7±3.4	26.2±3.1	24.9± 3.7
Psychosomatic response to stress	18.4±3.7	18.9±3.6	17.8±3.6
Social support	11.6±2.9	12.3±3.0	10.7±2.7
Distribution of 5HTT polymorphisms			
s/s	152	88 (63.8)	64 (61.0)
s/l	77	45 (32.6)	32 (30.5)
l/l	14	5 (3.6)	9 (8.6)
s frequency		0.80	0.76
l frequency		0.20	0.24
Distribution of ALDH2 polymorphisms			
*1/*1	138	84 (60.9)	54 (51.4)
*1/*2	94	48 (34.8)	46 (43.8)
*2/*2	11	6 (4.3)	5 (8.6)
*1 frequency		0.78	0.73
*2 frequency		0.22	0.27
Distribution of DRD2 polymorphisms			
A1/A1	36	18 (13.0)	18 (17.1)
A1/A2	100	58 (42.0)	42 (40.0)
A2/A2	107	62 (44.9)	45 (42.9)
A1 frequency		0.34	0.37
A2 frequency		0.66	0.63
Distribution of CYP2A6 polymorphisms			
Normal inactivator (n)	36	22 (15.9)	14 (13.3)
Intermediate inactivator (i)	80	43 (31.2)	37 (35.2)
Slow inactivator (s)	126	73 (52.6)	53 (50.5)
n frequency		0.32	0.31
s frequency		0.68	0.69

Scores for depressed mood, stressors, psychosomatic response to stress and social support were calculated with the Brief Job Stress Questionnaire. [a]Statistical differences were observed between males and females, $p<0.001$. All allele frequencies were calculated according to the Hardy-Weinberg law.

were calculated by logistic regression using Stat View (5.0, SAS Institute Inc., Cary, NC, USA).

Results

Lifestyle factors and polymorphisms of subjects. The mean age of the male subjects was almost the same as that of the female subjects (Table I). Although the ratio of drinkers was not significantly different between males and females, the average alcohol consumption of male drinkers was more than twice that of female drinkers. The average consumption of the male drinkers was also higher than the Japanese average, which is ~200 g/week (25). The ratio of smokers was lower in females than in males, as expected, but the ratios of both were higher than the Japanese averages (43% for males and 12% for females) (26). The mean Brinkman index for male smokers was approximately twice that of female smokers. Since lifestyle factors related to drinking and smoking were

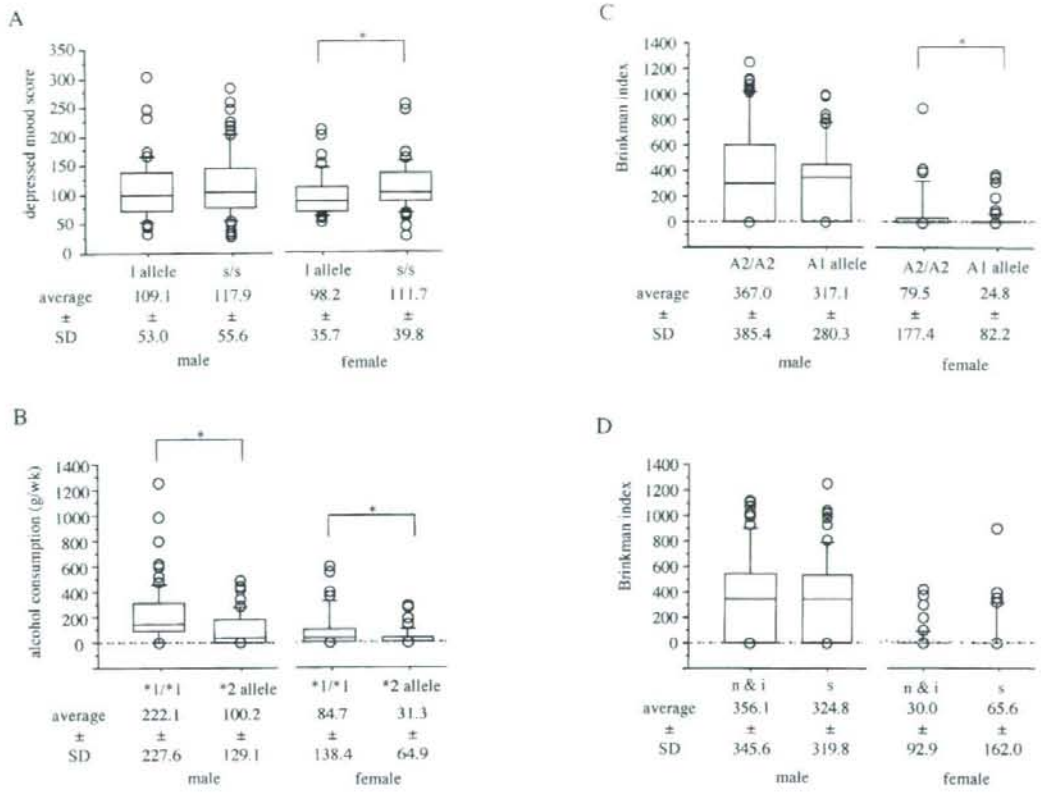

Figure 2. Relationship between lifestyle factors and each polymorphism for each gender (the Mann-Whitney U test was performed). (A) Relationship between depressed mood score and 5HTT polymorphisms. Depressed mood score in females carrying the s/s allele was significantly higher than in those with the l allele. (B) Relationship between alcohol consumption and ALDH2 polymorphisms. Carriers of the ALDH2 *2 allele showed significantly lower amounts of alcohol in both genders. (C and D) Relationships between the Brinkman index and DRD2 polymorphisms (C), and CYP2A6 polymorphisms (D). The DRD2 A1 allele group showed significantly lower Brinkman index than the A2/A2 group in females. *p<0.05.

Table II. Association between lifestyle factors and polymorphisms using factor analysis.

	Male			Female		
	Factor 1	Factor 2	Factor 3	Factor 1	Factor 2	Factor 3
Age	0.710	0.384		0.384		0.431
Gender						
Alcohol consumption (g/week)	0.572	-0.486	-0.334	0.750		
Brinkman index	0.777			0.738		
Depressed mood			0.616	0.472		-0.365
5HTT polymorphisms		-0.428	0.489	0.349	-0.668	
ALDH2 polymorphisms		0.670	0.408	-0.354		-0.494
DRD2 polymorphisms	-0.351					0.673
CYP2A6 polymorphisms		-0.450	0.501		0.729	
Eigenvalue	1.639	1.299	1.175	1.829	1.185	1.152
Contribution ratio	0.205	0.162	0.147	0.229	0.148	0.144
			p<0.001			p<0.01

Absolute factor loadings >0.3. Three factors were selected for totals and for each gender. Each polymorphism was represented as follows: 5HTT: l allele, 0; s/s, 1. ALDH2: *1/*1, 0; *2 allele, 1. DRD2: A2/A2, 0; A1 allele, 1. CYP2A6: normal and intermediate inactivators, 0; slow inactivator, 1.

Table III. Risk determinants in the increase of depressed mood estimated by logistic regression analysis.

	Male				Female			
	Odds ratio	95% CI	Odds ratio	95% CI	Odds ratio	95% CI	Odds ratio	95% CI
Age	1.01	0.97-1.06	1.01	0.96-1.06	0.97	0.93-1.02	1.00	0.94-1.06
Gender								
5HTT polymorphism								
s/s based on l allele	1.25	0.59-2.63	1.33	0.58-3.06	3.36[b]	1.15-9.76	2.26	0.58-8.82
ALDH2 polymorphism								
*2 allele based on *1/*1	0.63	0.30-1.36	0.61	0.26-1.41	0.76	0.29-1.99	0.43	0.12-1.50
DRD2 polymorphism								
A1 allele based on A2/A2	1.06	0.52-2.16	1.53	0.68-3.44	1.27	0.48-3.36	2.14	0.58-7.88
CYP2A6 polymorphism[a]	1.41	0.69-2.88	1.15	0.51-2.59	1.73	0.66-4.53	1.66	0.49-5.65
Alcohol consumption (g/week)	0.999	0.997-1.001	0.999	0.996-1.001	1.000	0.995-1.004	1.000	0.995-1.004
Brinkman index	1.000	0.99-1.002	1.000	0.99-1.002	1.005[b]	1.000-1.009	1.003	0.998-1.009
Social support			0.07[c]	0.02-0.21			0.03[c]	0.01-0.13

Odds ratios indicating that depressed mood was higher than the mean score were calculated according to potential risk factors. [a]CYP2A6 polymorphism: slow inactivator based on normal and intermediate inactivators. [b]$p<0.05$, [c]$p<0.0001$.

different between males and females, further analysis was performed on the basis of gender. There were no gender differences with regard to stressors, psychosomatic response to stress and social support estimated by the Brief Job Stress Questionnaire (17). No statistical difference was observed in depressed mood, while the depressed mood score in males was slightly higher than that in females, both of which were higher than those of the national mean (of 100) in Japan (17).

There was no gender difference in the allele frequencies of the gene polymorphisms, i.e. all the polymorphisms examined were in agreement with the Hardy-Weinberg law in both genders. In the 5HTT polymorphisms, the allele frequencies were s, 0.80 and l, 0.20 in males, and s, 0.76 and l, 0.24 in females. Since the number of l/l homozygotes was very small, the 5HTT group was subdivided into s/s and l allele groups. Similarly, in the ALDH2 polymorphisms, allele frequencies were *1, 0.78 and *2, 0.22 in males, and *1, 0.73 and *2, 0.27 in females. Consequently, the ALDH2 group was subdivided into *1/*1 and *2 allele groups. The DRD2 polymorphisms included allele frequencies A1, 0.34 and A2, 0.66, and A1, 0.37 and A2, 0.63, for males and females, respectively. The DRD2 polymorphisms were therefore analyzed for A1 and A2/A2 allele groups. The observed distributions of these genotypes were in good agreement with previous reports (5,6,23,27). The ratios of n, i, and s CYP2A6 polymorphisms were 0.32 and 0.68 in both genders. Since low cigarette consumption has been reported in carriers of the s polymorphism (24), the effect of s was assessed against n and i.

Univariate comparison between lifestyle factors and polymorphisms. The differences of depressed mood score and lifestyle factors in each polymorphism group were examined. Significantly higher depressed mood score was observed only in females carrying the s/s allele of 5HTT rather than the l/l and l/s alleles (Fig. 2A). The ALDH2, DRD2, and CYP2A6 groups had no significant differences in depressed mood score regardless of gender (data not shown). Alcohol consumption of the ALDH2 *1/*1 allele group was higher than that of the ALDH2 *2 allele group irrespective of gender (Fig. 2B), because ALDH2*2 lacked activity for acetaldehyde metabolism. However, the alcohol consumption of the male ALDH2 *2 allele group was almost the same as that of the female ALDH2 *1/*1 allele group. No statistically significant differences were observed between drinking habits and 5HTT, DRD2, or CYP2A6 polymorphisms irrespective of gender (data not shown). Although DRD2 and CYP2A6 polymorphisms have been associated with smoking (7), these polymorphisms did not affect males (Fig. 2C and D). In females, however, the DRD2 A1 allele group showed a significantly lower Brinkman index than the A2/A2 group. Multivariate analyses such as factor analysis and logistic regression analysis were further applied in order to remove possible confounding factors and to clarify the risks of depressed mood score.

Multivariate analysis to determine the risk of depressed mood score. Age, gender, alcohol consumption, the Brinkman index, depressed mood score, and polymorphisms of the 5HTT, ALDH2, DRD2, and CYP2A6 genes were selected as a determinant for factor analysis (Table II). For both genders, this analysis allowed us to select three factors with statistically significant differences. The contribution ratio of each factor was almost the same between males and females, but the determinants constituting each factor differed. In males, factor 1 was defined as the lifestyle factor because age, alcohol consumption and the Brinkman index showed strong correlations, whereas DRD2 polymorphism was also included. Factor 2 was defined as the alcohol factor because age, alcohol consumption, and 5HTT, ALDH2 and CYP2A6 polymorphisms were correlated. Factor 3 was defined as the

stress factor because depressed mood was strongly correlated with 5HTT polymorphisms. In addition, ALDH2 polymorphisms and alcohol consumption showed positive and negative correlations, respectively. The 5HTT s/s allele was associated with a high depressed mood score. In females, factor 1 was defined as the stress factor because of a positive correlation among age, alcohol consumption, Brinkman index, depressed mood, and 5HTT polymorphisms, and a negative correlation of ALDH2 polymorphisms. Since depressed mood score and 5HTT polymorphisms appeared as factor 3 in males, their contribution ratio in female was higher than in males.

As the average score for depressed mood was ~110 in all subjects, a logistic regression analysis was performed to determine possible risk factors which increased depressed mood score to >110. Logistic regression analysis indicated that, only in females, did the s/s polymorphism of 5HTT increase the risk of a higher depressed mood score compared with the l allele, of which the odds ratio was 3.36 (Table III). The odds ratio was low but a high Brinkman index was also recognized as a risk in females. Polymorphisms for ALDH2, DRD2, and CYP2A6 were not risks for the increase in depressed mood score for either gender. When social support was included as a determinant, it was found to reduce depressed mood score in both genders. Moreover, in females, this protective effect was independent of 5HTT polymorphisms.

Discussion

Job stress is one of the major risk factors for depression. The Brief Job Stress Questionnaire used in the present study characterizes many aspects of stress. Firstly, not only the stress reactions of workers but also stressors in the workplace can be estimated. Secondly, not only negative but also positive psychological reactions can be estimated. Thirdly, it uses multi-axis methods to assess somatic reactions and modifying factors as well as these psychological reactions (17). This questionnaire clarifies the three major factors of job stress, i.e. stressors, psychosomatic responses to stress, and social support. Stressors include age, gender, quantitative, qualitative and somatic workload, job control, interpersonal relations, work environment and job fitness. Psychosomatic responses to job stress consist of lack of vigor, irritability, fatigue, anxiety, depressed mood, and somatic symptoms. Social support consists of supervisor, coworker and family support. Excessive stressors can cause work-related diseases, and social support can ameliorate these conditions. This questionnaire does not consider factors of family life or personality. In the present study, we focused on depressed mood among psychosomatic responses, because depressed mood is considered to be the highest correlation coefficient to work-related stressful conditions using covariance structure analysis (17). The mean level of depressed mood of our subjects was higher than that of a previous study in Japan (17), which may indicate that the subjects in the present study work under high job stress conditions.

Univariate analysis showed the relationships between depressed mood score and 5HTT polymorphisms. The short (s) allele in the 5HTT gene is associated with lower serotonin re-uptake compared with the long (l) allele. This polymorphism is associated with many diseases or dependencies such as anxiety (27), suicide (28), smoking (29) and alcohol dependence (30), and attention deficit hyperactivity disorder (ADHD) (31). Distribution of the l/l homozygote in Japanese is ~4% (27), which is lower than in Caucasians (~30%) (19). Moreover, distribution of the s/s homozygote in Japanese is ~60%, which is higher than in Caucasians (~20%). The distribution of 5HTT polymorphisms was almost the same as this previously reported distribution (27). An epidemiological study indicated that individuals with the s/s homozygote of 5HTT exhibit depressive symptoms, diagnosable depression, or suicidality associated with very stressful life events including employment problems (long-term unemployment, employer bankruptcy, lay-off, termination), financial problems (debt, inadequate funds for living expenses), housing problems (homelessness, multiple residence changes), health problems (disabling physical illness lasting a month or more, disabling injury), or relationship problems (death of a family member, being in a physically violent relationship, break-up of a cohabitation relationship) (19). According to the Mann-Whitney U test, a high-depressed mood score in carriers of the s/s allele of 5HTT was only found in female subjects; this was also confirmed by factor and logistic regression analyses. These results indicate that the 5HTT s/s allele in female subjects was recognized to be a potential risk factor for depressed mood caused by job stress, which was commonly observed in the workplace. This might indicate that females were more sensitive to stress.

Although the 5HTT s/s allele was a risk factor for depressed mood, social support reduced this risk. A previous study (32) showed that individuals who had the 5HTT s/s allele had greater depressive symptomatology if they had experienced early or recent adversity but significantly less depressive symptomatology if they reported a supportive early environment or recent positive experiences, compared with individuals with the s/l or l/l genotype. In the present study, a depressed mood score was reduced by social support irrespective of 5HTT polymorphisms and gender. This result indicated that the 5HTT genotype did not necessarily predetermine the incidence of depressed mood caused by job stress. Although gene-by-environment interaction might affect the incidence of depressed mood, environmental factors such as adequate support might be more important to prevent the incidence of depressed mood.

Alcohol consumption by subjects with the ALDH2 *2 allele was significantly lower than in those with the ALDH2 *1/*1 allele, since the ALDH2 polymorphism is associated with drinking behavior (5). Individuals with the ALDH2 *2/*2 homozygous allele are not able to metabolize alcohol, and individuals with the ALDH2 *1/*2 heterozygous allele experience facial flushing in response to alcohol (33). While ~65% of subjects had the ALDH2 *1/*1 homozygous allele, a distribution that is typical of Japanese, most of them were able to drink alcohol. Logistic regression analysis showed no relationship between depressed mood and alcohol consumption. On the other hand, male subjects consumed more alcohol than females. Even when carrying the ALDH2 *2 allele, their alcohol consumption was comparable to that of the female ALDH2 *1/*1 allele group. It is likely that the

relaxation effect of alcohol might conceal the effect of the 5HTT s/s allele in the case of the male subjects. Since dopamine D2 receptor and CYP2A6 polymorphisms were associated with smoking status (6,22), non-parametric tests showed that the Brinkman index of the female DRD2 A1 allele group was lower than that of the A2/A2 group. Moreover, logistic regression analysis showed the Brinkman index corresponded with the risk of increasing depressed mood, especially in female subjects. Although Cinciripini *et al* (22) demonstrated that smokers carrying the A1 allele showed no reduction in negative mood during antidepressant therapy, our results indicated no relationship between depressed mood and DRD2 polymorphisms. Further investigation is needed in order to clarify the effects of the A1 allele.

Diagnosable depression is associated not only with environmental factors but also genetic factors. Depressed mood shown in the present study did not present as a disease, but as a response to job stress. Although the present study involved only 241 subjects, an association of depressed mood with a polymorphism of 5HTT was observed. Since Japanese have higher rates of the s/s allele compared to Caucasians, the reduction of job stress by social support might be effective in reducing depressed mood. Further research is needed to corroborate a relationship between mental disorders and environmental and genetic factors.

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References

1. Malkin D, Li FP, Strong LC, *et al*: Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. Science 250: 1233-1238, 1990.
2. Peter I, Shearman AM, Vasan RS, *et al*: Association of estrogen receptor beta gene polymorphisms with left ventricular mass and wall thickness in women. Am J Hypertens 18: 1388-1395, 2005.
3. Katsuyama H, Ideguchi S, Fukunaga M, Saijoh K and Sunami S: Usual dietary intake of fermented soybeans (Natto) is associated with bone mineral density in premenopausal women. J Nutr Sci Vitaminol 48: 207-215, 2002.
4. Tadokoro K, Hashimoto R, Tatsumi M, Kosuga A, Kamijima K and Kunugi H: The Gem interacting protein (GMIP) gene is associated with major depressive disorder. Neurogenetics 6: 127-133, 2005.
5. Okamoto K, Murawaki Y, Yuasa I and Kawasaki H: Effect of ALDH2 and CYP2E1 polymorphisms on drinking behavior and alcoholic liver disease in Japanese male workers. Alcohol Clin Exp Res 25: 19s-23s, 2001.
6. Nakajima M, Kwon JT, Tanaka N, *et al*: Relationship between interindividual differences in nicotine metabolism and CYP2A6 genetic polymorphism in humans. Clin Pharmacol Ther 68: 72-78, 2001.
7. Spitz MR, Shi H, Yang F, *et al*: Case-control study of the D2 dopamine receptor gene and smoking status in lung cancer patients. J Natl Cancer Inst 90: 358-363, 1998.
8. Hariri A, Mattay VS, Tessitore A, *et al*: Serotonin transporter genetic variation and the response of the human amygdala. Science 297: 400-403, 2002.
9. Harada S and Zhang S: New strategy for detection of ALDH2 mutant. Alcohol Alcohol 28: S11-S13, 1993.
10. Nagata S: Stress management in the workplace in the era of industrial and economic change. Sangyo Eiseigaku Zasshi 42: 215-220, 2000 (in Japanese).
11. Karasek R, Baker D, Marxer F, Ahlbom A and Theorell T: Job decision latitude, job demands, and cardiovascular disease: a prospective study of Swedish men. Am J Public Health 71: 694-705, 1981.
12. Wells KB, Steward A and Hays RD: The functioning and well-being of depressed patients. JAMA 264: 914-919, 1989.
13. Nakata A, Haratani T, Takahashi M, *et al*: Job stress, social support at work, and insomnia in Japanese shift work. J Hum Ergol 30: 203-209, 2001.
14. Brady KT and Sinha R: Co-occurring mental and substance use disorders: the neurobiological effects of chronic stress. Am J Psychiatry 162: 1483-1493, 2005.
15. Karasek R: Job Content Questionnaire. Department of Industrial and Systems Engineering, University of Southern California, Los Angeles, 1985.
16. Shatchkute A: MONICA Optional Psychosocial Substudy (MOPSY). In: MONICA Monograph and Multimedia Sourcebook. Tunstall-Pedoe H (ed). WHO, pp86-87, 2003.
17. Shimomitsu T, Haratani T, Nakamura K, *et al*: The final development of the Brief Job Stress Questionnaire mainly used for assessment of the individuals. In: Ministry of Labour Sponsored Grant For the Prevention of Work-Related Illness: The 1999 Report. Kato M (ed). Tokyo Medical College, Tokyo pp126-164, 2000 (in Japanese).
18. Brinkman GL and Coates EO Jr: The effect of bronchitis, smoking, and occupation on ventilation. Am Rev Respir Dis 87: 684-693, 1963.
19. Caspi A, Sugden K, Moffitt TE, *et al*: Influence of life stress on depression: Moderation by a polymorphism in the 5-HTT gene. Science 301: 386-389, 2003.
20. Weiss R and Rompre P: Brain dopamine and reward. Annu Rev Psychol 40: 191-225, 1989.
21. Corrigall WA, Franklin KBJ, Coen KM and Clarke PBS: The mesolimbic dopaminergic system is implicated in the reinforcing effects of nicotine. Psychopharmacology 107: 285-289, 1992.
22. Cinciripini PM, Wetter DW, Tomlinson GE, Tsoh JY, De Moor CA, Cinciripini LG and Minna JD: The effects of the DRD2 polymorphism on smoking cessation and negative affect: Evidence for a pharmacogenetic effect on mood. Nicotine Tob Res 6: 229-239, 2004.
23. Matsushita S, Muramatsu T, Murayama M, Nakane J and Higuchi S: Alcoholism, ALDH2*2 allele and the A1 allele of the dopamine D2 receptor gene: an association study. Psychiatry Res 104: 19-26, 2001.
24. Schoedel KA, Hoffmann EB, Rao Y, Sellers EM and Tyndale RF: Ethnic variation in CYP2A6 and association of genetically slow nicotine metabolism and smoking in adult Caucasians. Pharmacogenetics 14: 615-626, 2004.
25. Okamoto E: Public Health and Preventive Medicine, Subnote 30th edition. Okaniwa Y (ed). Medic Media p154, 2007.
26. Health and Welfare Statistics Association: J Health Welfare Stat. Health and Welfare Statistics Association, 2005 (in Japanese).
27. Katsuragi S, Kunugi H, Sano A, Tsutsumi T, Isogawa K, Nanko S and Akiyoshi J: Association between serotonin transporter gene polymorphism and anxiety-related traits. Biol Psychiatry 45: 368-370, 1999.
28. Hranilovic D, Stefulj S, Furac I, Kubat M, Balija M and Jernej B: Serotonin transporter gene promoter (5-HTTLPR) and intron 2 (VNTR) polymorphisms in Croatian suicide victims. Biol Psychiatry 54: 884-889, 2003.
29. Ishikawa H, Ohtsuki T, Ishiguro H, *et al*: Association between serotonin transporter gene polymorphism and smoking among Japanese males. Cancer Epidemiol Biomarkers Prev 8: 831-833, 1999.
30. Ishiguro H, Saito T, Akazawa S, *et al*: Association between drinking-related antisocial behavior and a polymorphism in the serotonin transporter gene in Japanese population. Alcohol Clin Exp Res 23: 1281-1284, 1999.
31. Kent L, Doerry U, Hardy E, *et al*: Evidence that variation at the serotonin transporter gene influences susceptibility to attention deficit hyperactivity disorder (ADHD): Analysis and pooled analysis. Mol Psychiatry 7: 908-912, 2002.
32. Taylor SE, Way BM, Welch WT, Hilmert CJ, Lehman BJ and Eisenberger NI: Early family environment, current adversity, the serotonin transporter promoter polymorphism, and depressive symptomatology. Biol Psychiatry 60: 671-676, 2006.
33. Yokoyama T, Yokoyama A, Kato H, *et al*: Alcohol flushing, alcohol and aldehyde dehydrogenase genotypes, and risk for esophageal squamous cell carcinoma in Japanese men. Cancer Epidemiol Biomarkers Prev 12: 1227-1233, 2003.

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Changes in salivary physiological stress markers associated with winning and losing

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ABSTRACT

Using a representative table game popular in Japan known as *shogi*, or Japanese chess, we investigated the effects of winning and losing on saliva composition. The subjects were 90 healthy male university students who were members of a *shogi* club. Saliva samples were collected immediately before and after playing *shogi*, and again 30 min later. Salivary cortisol and testosterone levels in the samples were determined by ELISA and EIA, respectively. After finishing each game, the competitiveness of the game was evaluated using questionnaires. In the samples taken after playing *shogi*, there was an increase in the levels of salivary testosterone and cortisol, regardless of whether the subject won or lost, and the tendency was more pronounced in competitive games. There were no such changes in the control group, who did not play a game prior to providing the samples. Our results suggest that stress response is intimately linked with competition and could be used to determine which players are more capable of handing stress in a competitive environment.

There are a number of confrontational situations that occur in nature. Of those, competition involves two or more individuals struggling for mastery while attempting to achieve the same goal. Thus, along with winners there are also losers. Previous research indicates that sports and gambling participation induce stress (3, 14). However, those studies did not investigate the interactions between winners and losers, and how competitive stress triggers physiological symptoms has not been revealed.

McCaul (13) reported greater increases of testosterone in saliva and more positive moods in winners than losers among individuals who participated in a task entirely controlled by chance (coin tossing). In another experiment (11), subjects were awarded $100 prizes depending on a random lottery. Winners in those situations, who won without any effort of their own, did not show subsequent testosterone increases in blood that were greater than those of the losers. These experiments, conducted in a casual manner, do not necessarily indicate that the derived conclusion reflects stress reaction.

Real competition, especially related to sports, has been well researched in psychoneurotic endocrinological studies, though universal agreement on a number of factors has not been reached. For example, one study reported that levels of testosterone and cortisol in blood samples from winning wrestlers rose higher above the level of the previous match as compared to the losing wrestlers (15), whereas another study reported that those levels were not different between winners and losers (5). The reason for this discrepancy may be related to physical stress caused by participation in sporting events. In fact, those stress measurements may have been affected by psychological stress induced by competition.

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In the present investigation, we studied subjects who participated in a table game popular in Japan known as *shogi*, or Japanese chess, in order to exclude the effects of physical stress from the environment of competition, and measured the levels of testosterone and cortisol in saliva samples. The competitiveness of *shogi* is partly derived from its comparatively long period of playing, thus we considered it an effective means to study psychological stress. In addition, we reviewed the competitive aspects of each game, to determine the intensity of the competition.

MATERIALS AND METHODS

The subjects were 90 healthy male students, who were members of the *shogi* club at Osaka University and participating in the Western Japan Convention hosted by the Kansai Student Shogi Association. None were smokers or taking any medications. Forty one of the subjects (mean ± SD, 21.3 ± 2.7 years old) played *shogi* during the convention (shogi group) and 49 (19.5 ± 2.0 years old) watched the games (control group). All subjects gave consent to participate in the study, after being informed of its purpose and method. Saliva samples were collected from both groups immediately before and after the games were played, and again 30 min later (Fig. 1) using a 50-mL polypropylene conical tube (BLUE MAX™). The subjects were requested to refrain from eating and drinking at least 2 h before the first sampling (19). The samples were stored at −30°C until the assay. Salivary cortisol levels were determined by ELISA (enzyme-linked immunosorbent assay) and testosterone levels by EIA (enzyme immunoassay), using previously described methods (7, 8, 18). Following each game, the shogi group reported whether the game was competitive or non-competitive. Values were converted to percentages of the baseline (before playing *shogi*). ANOVA with repeated measures was performed to examine time-related differences and Bonferroni's test was used for multiple comparisons. Values were considered to be significantly different at $p < 0.05$.

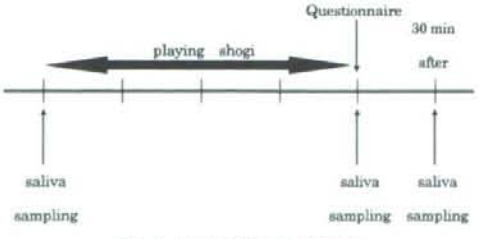

Fig. 1 Experimental protocol

RESULTS

In samples from the shogi group taken after playing the game, there were significant increases in the levels of salivary testosterone (63.8 ± 21.7 to 73.2 ± 20.9 pg/mL; $p < 0.01$) and cortisol (0.446 ± 0.281 to 0.800 ± 0.354 μg/dL; $p < 0.05$) (Fig. 2), though the increased levels were not maintained in the samples taken 30 min later (testosterone and cortisol levels were 63.7 ± 17.2 pg/mL and 0.473 ± 0.251 μg/dL, respectively). As for the control group, there were no significant changes in the levels of cortisol and testosterone. We categorized the samples taken from the shogi group into 2 sub-groups, winners and losers, based on the outcome of the game, however, found was no significant difference in salivary tes-

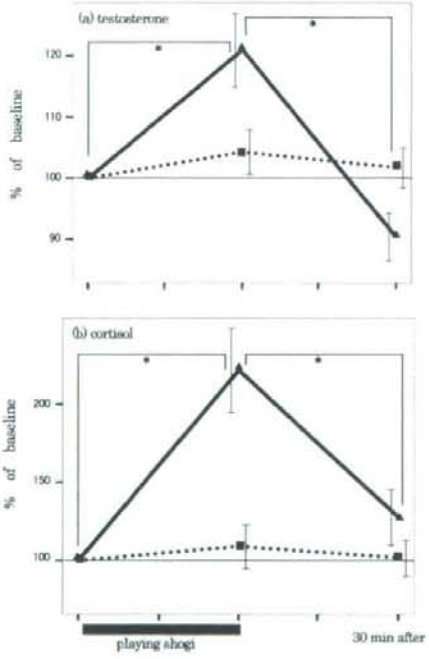

Fig. 2 Mean values (± SE) are presented as percentages of the baseline values obtained before playing a game. (a) Salivary testosterone and (b) cortisol in samples taken from the shogi (▲, n = 41) and control (■, n = 49) groups. *Significantly different ($p < 0.05$) as determined by repeated measures ANOVA and Bonferonni's test.

tosterone level between the sub-groups (Fig. 3). However, the increase seen in the winners had a relatively greater amount of reduction in the samples taken 30 min later. Salivary cortisol showed the same tendency, and the difference was not significant. We also categorized the samples taken from the shogi group into 2 sub-groups, competitive and non-competitive, according to the questionnaire results. In the samples taken following a competitive game, there were significant increases in the levels of salivary testosterone (66.0 ± 23.9 to 76.8 ± 20.7 pg/mL; $p < 0.01$) and cortisol (0.477 ± 0.305 to 0.880 ± 0.380 μg/dL; $p < 0.05$) (Fig. 4), whereas there were no such changes in the samples taken following a non-competitive game.

DISCUSSION

Rising levels of salivary testosterone and cortisol were identifiable in samples taken from the shogi group, whereas those from the control group were considered to reveal no effect from the game on the endocrine system. It has been reported that the perceptions of winning and losing regardless of actual performance or merit had different influences on testosterone levels. In a previous study, testosterone levels showed increases and decreases in fans, depending on the outcome of their team (2). For this reason, we expected that the levels of testosterone in samples from the control group would change, however, we were not able to confirm such results. We attributed this to the fact that the present control group differed in a number of ways from fans cheering for their team.

As for the participants, Mazur *et al.* (12) compared samples taken following competitive and non-competitive chess games, and found that the levels of salivary testosterone were significantly different. Our results (Fig. 4) confirmed those and suggested that stress response is intimately linked with com-

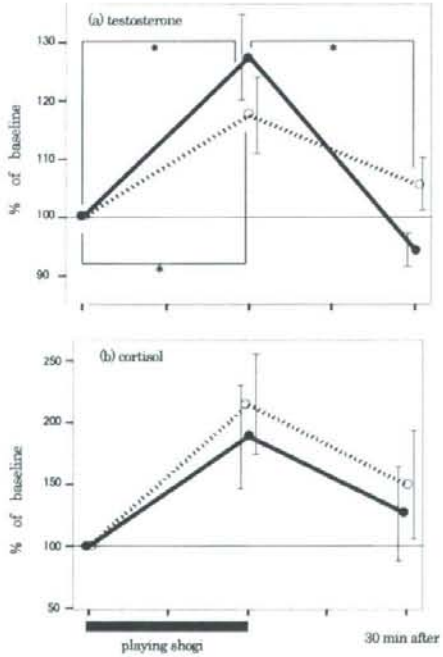

Fig. 3 Mean values (± SE) are presented as percentages of the baseline values obtained before playing a game. (a) Salivary testosterone and (b) cortisol levels were measured in the winners (●, n = 15) and losers (○, n = 26). *Significantly different ($p < 0.05$), as determined by repeated measures ANOVA and Bonferonni's test.

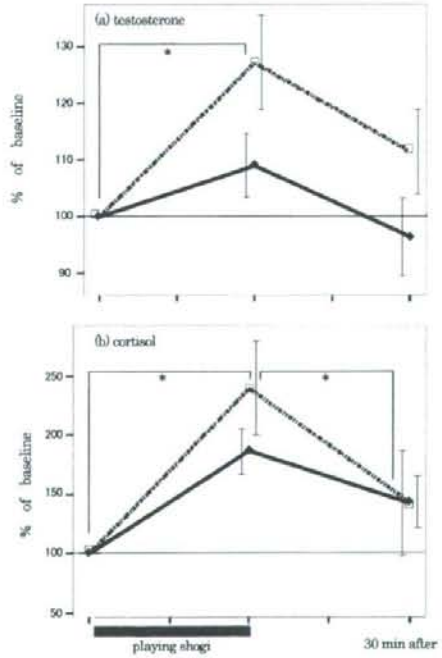

Fig. 4 Mean values (± SE) are presented as percentages of the baseline values obtained before playing a game. (a) Salivary testosterone and (b) cortisol levels in samples taken following non-competitive (◆, n = 14) and competitive (□, n = 27) games. *Significantly different ($p < 0.05$), as determined by repeated measures ANOVA and Bonferonni's test.

petitiveness, based on our assumption that the sense of stress following a competitive game would continue 30 min later. On the other hand, in a non-competitive game, players are able to anticipate the result before the game is finished, resulting in a lower sense of stress, because of feeling of resignation from defeat or relief from victory toward the end of the game. Thus, the stress responses of participants are inhibited during a non-competitive game.

In the shogi group, there were no differences in levels of salivary testosterone and cortisol between the winners and losers. This supports a previous report (16), which found that there were no differences in samples collected from male judo wrestlers in regard to the levels of testosterone and cortisol in blood taken before and after a match. Some studies have suggested that testosterone levels are related to positive coping behavior (9, 10). That is, positive coping behavior in individuals sensing stress causes elevated levels of testosterone and activity of the sympathetic nervous system. In addition, when an individual has self-confidence and experiences positive emotions, they tend to view stressful situations as challenging (2, 4, 6). Therefore, in the present study, it is possible that the winners demonstrated positive coping behavior and the losers negative coping behavior. It is important for future studies to examine the relationships among the endocrine system and a number of factors that influence stress response, such as lifestyle, personality, and stress coping behavior. Additionally, because the mechanism for such a testosterone effect is unclear even in non-human animals (17), we intend to reveal it in future studies.

REFERENCES

1. Bandura A (1991) Self-efficacy mechanism in physiological activation and health promoting behavior. In: *Neurobiology of Learning, Emotion, and Affect* (Mudden J, ed.), pp229–269, Raven Press, New York.
2. Bernhardt PC, Dabbs Jr. JM, Fielden JA and Lutter CD (1998) Testosterone changes during vicarious experiences of winning and losing among fans at sporting events. *Physiol Behav* **65**, 59–62.
3. Chatterton Jr. RT, Vogelsong KM, Lu YC and Hudgens GA (1997) Hormonal responses to psychological stress in men preparing for skydiving. *J Clin Endocrinol Metab* **82**, 2503–2509.
4. Dienstbier RA (1989) Arousal and physiological toughness: implications for mental and physical health. *Psychol Rev* **96**, 84–100.
5. Elias M (1981) Serum cortisol, testosterone, and testosterone-binding globulin responses to competitive fighting in human males. *Aggressive Behav* **7**, 215–224.
6. Eubank M, Collins D, Lovell G, Dorling D and Talbot S (1997) Individual temporal differences in precompetition anxiety and hormonal concentration. *Person Individ Diff* **23**, 1031–1039.
7. Granger DA, Schwartz EB, Booth A and Arentz M (1999) Salivary testosterone determination in studies of child health and development. *Horm Behav* **35**, 18–27.
8. Granger DA, Shirtcliff EA, Booth A, Kivlighan KT and Schwartz EB (2004) The "trouble" with salivary testosterone. *Psychoneuroendocrinology* **29**, 1229–1240.
9. Henry JP (1986) Neuroendocrine patterns of emotional response. In: *Emotion: Theory, Research and Experiences* (Plutchik R and Kellerman H, eds.), pp37–60, Academic Press, San Diego.
10. Koolhaas JM, Korte SM, De Boer SF, Van Der Vegt BJ, Van Reenen CG, Hopster H, De Jong IC, Ruis MA and Blokhuis HJ (1999) Coping styles in animals: current status in behavior and stress-physiology. *Neurosci Biobehav Rev* **23**, 925–935.
11. Mazur A and Lamb TA (1980) Testosterone, status and mood in human males. *Horm Behav* **14**, 236–246.
12. Mazur A, Booth A and Dabbs JM (1992) Testosterone and chess competition. *Social Psychol* **55**, 70–77.
13. McCaul KD, Gladue BA and Joppa M (1992) Winning, losing, mood, and testosterone. *Horm Behav* **26**, 486–504.
14. Meyer G, Hauffa BP, Schedlowski M, Pawlak C, Stadler MA and Exton MS (2000) Casino gambling increases heart rate and salivary cortisol in regular gamblers. *Biol Psychiatry* **48**, 948–953.
15. Passelergue P and Lac G (1999) Saliva cortisol, testosterone and T/C ratio variations during a wrestling competition and during the post-competition recovery period. *Int J Sports Med* **20**, 109–113.
16. Salvador A, Simon V, Suay F and Llorens L (1987) Testosterone and cortisol responses to competitive fighting in human males: A pilot study. *Aggressive Behav* **13**, 9–13.
17. van Anders SM and Watson NV (2007) Effects of ability- and chance-determined competition outcome on testosterone. *Physiology Behavior* **90**, 634–642.
18. Shimada M, Takahashi K, Ohkawa T, Segawa M and Higurashi M (1995) Determination of salivary cortisol by ELISA and its application to the assessment of the circadian rhythm in children. *Horm Res* **44**, 213–217.
19. Toda M, Morimoto K, Nagasawa S and Kitamura K (2004) Effect of snack eating on sensitive salivary stress markers cortisol and chromogranin A. *Environ Health Prev Med* **9**, 27–29.

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Adequacy of continuation and maintenance treatments for major depression in Japan

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Abstract

Guidelines for treating depression often recommend continuing antidepressants at least for 6 months after remission. Whether this recommendation is implemented in daily practices represents a serious concern. We aimed to examine adequacy of continuation and maintenance treatment in Japan. A naturalistic prospective follow-up study with mood disorders was undertaken in 23 psychiatric departments from all over Japan. A total of 95 patients diagnosed with major depression were followed up every month until treatment termination and every 6 months thereafter. In this study, the cohort received 45.1 (SD = 64.7) mg of imipramine or equivalent per day during continuation phase, and about 74% were prescribed inadequate doses, i.e. less than 75 mg/day. At maintenance phase immediately before relapse, average dosage was 42.0 (SD = 74.7) mg/day and 83% were prescribed inadequate doses. There is gross undertreatment of depression during continuation and maintenance phases in Japan.

Keywords

major depressive disorder, continuation treatment, maintenance treatment

Introduction

The treatment of a depressive episode can be conceptualized into acute phase, continuation and maintenance treatment (Kupfer, 1991). Following this scheme, Frank *et al.* (1991) defined recovery from a major depressive episode as consecutive 2–6 months with no more than one or two mild depressive symptoms, and called these consecutive 2–6 months as continuation phase, and the months following this continuation phase until the recurrence as maintenance phase.

Various guidelines have aimed at improving treatment in practice, because the risk of depressive relapse is significantly associated with discontinuing antidepressants soon after remission. Depression Guideline Panel (1993) recommended that continuation therapy should be given at full therapeutic dosage, i.e. 75–300 mg of Imipramine equivalent/day for 4–9 months. British Association for Psychopharmacology guideline (Anderson *et al.*, 2000) insists that after acute phase pharmacological treatment, patients should be prescribed the same dosage during continuation therapy for at least 6 months. A recent systematic review and meta-analysis (Geddes *et al.*, 2003) has conclusively demonstrated the importance of continuation and maintenance treatments finding that antidepressants continuation can halve the rates of relapse and recurrences after remission.

Although the model of longer-term treatment of depression has changed considerably in recent years with wider use of maintenance medication and greater use of psychological treatments (Fava *et al.*, 2004; Paykel *et al.*, 1999), there are few naturalistic studies

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on the treatment actually received by depressed patients during continuation and maintenance phases.

The Group for Longitudinal Affective Disorders Study (GLADS) has undertaken a detailed study of treatment received over 10 years, of a representative sample of depressed patients originally recruited in 1992–1995. The present report aims to assess adequacy of continuation and maintenance treatments in patients with major depression.

Materials and method

The study methods are described in detail elsewhere (Furukawa *et al.*, 2000a; Kanai *et al.*, 2003) and are summarized here. We conducted detailed prospective serial assessments of a cohort of patients with broadly defined affective disorders under naturalistic conditions. The 23 collaborating centers included psychiatric departments of 13 university hospitals and six general hospitals, three mental hospitals and one community mental health center from all over Japan. Participating psychiatrists at each center administered a semi-structured interview called the Psychiatric Initial Screening for Affective Disorders (PISA) (Kitamura, 1992) to its first-visit patients in order to ascertain the patients' eligibility. The eligibility criteria were:

1. depressive state, defined as presenting with depressed mood or anhedonia lasting longer than 4 days, or manic state, defined as presenting with elated, expansive or irritable mood lasting longer than 4 days;
2. having received no antidepressant or antipsychotic medication in the preceding 3 months;
3. aged 18 years or over;
4. absence of condition such as mental retardation, dementia or hearing disability, which would render detailed psychopathological assessment difficult.

Each participating centre was expected to enter one patient who satisfied the inclusion criteria either every month or every two months, depending on the availability of human and logistic resources at each centre, in order to avoid seasonable imbalance.

Written informed consent was obtained from all participants after full disclosure of the purposes and procedures of the study. The patients eligible for and consenting to the study were then interviewed within 1 week of entry by a psychiatrist using the entry version of the Comprehensive Assessment List for Affective Disorders (COALA) (Furukawa, 1992). The COALA consists of a series of semi-structured interviews that enable serial assessment of the cohort; these include the entry version, monthly follow-up version, and 6-monthly follow-up version. The reliability of the PISA and COALA has been reported to be good to excellent (Furukawa *et al.*, 1995). Quantitative assessments of drug treatment doses were made monthly while treatment lasted and 6-monthly thereafter up to 10 years. Monthly global ratings of depression severity were recorded.

In this study, we defined recovery from a major depressive episode as consecutive 6 months with no more than one or two mild depressive symptoms, because the CDS definition of recovery by 2 months of remission has been criticized for being too short (Tharyan and Raghuthaman, 1999). We defined these consecutive 6 months as continuation phase, and the months following this continuation phase until the recurrence as maintenance phase, and we investigated the actual daily dosage prescribed to the patients during this continuation phase and that at the end of the maintenance phase before the relapse. In order to determine adequacy of antidepressant dosage, we followed the Depression Guideline Panel (1993) and Furukawa *et al.* (2002) and considered imipramine equivalent more than 75 mg/day as adequate dosage. The equivalence of antidepressants was calculated in accordance with WHO Defined Daily Dosage (Tansella and Micciolo, 1992) by equating the average daily dosage of the preparation recommended for its main indication in adults. In Japan the first SSRI was not marketed until 1999 and all the antidepressants used for continuation or maintenance treatments during the period of this study were heterocyclics. We therefore chose to express dosage equivalence in terms of imipramine. The GLADS Project is a naturalistic follow-up study and there was no control over the treatment in its protocol. We used the statistical package SPSS for Windows 12.0 (SPSS Inc.).

Results

During the period between December 1992 and December 1995, 1968 patients were screened at the 23 participating centres. Out of those, 126 patients, who had been selected according to prespecified rules to avoid seasonal imbalance and who had gave their written informed consent, were formally entered into the study and have now been followed up to 10 years. The 126 patients were not different from the rest of the larger pool of patients ($n = 916$) who satisfied the eligibility criteria but were not entered into the study in terms of age ($t = -0.59$, df = 1014, $p = 0.56$), sex ($\chi^2 = 0.58$, df = 1, $p = 0.81$) or season of entry ($\chi^2 = 1.90$, df = 3, $p = 0.59$).

The diagnoses of these 126 subjects according to DSM-IV were major depressive disorder ($n = 95$), depressive disorder NOS (15), bipolar I disorder (7), bipolar II disorder (3) and others (6). In the following analysis, we will concentrate on the 95 subjects who were diagnosed with major depressive disorder according to DSM-IV (single episode, 67; recurrent, 28). Nine had other axis I comorbid disorders: panic disorder (3), generalized anxiety disorder (2), social phobia (1), anorexia nervosa (1), alcohol intoxication (1) and vascular dementia (1). Fourteen of them (15%) were in-patients at the time of the administration of the COALA Entry version.

Among these 95 patients, 84 reached recovery, defined as 6 months of remission, 10 were lost to follow-up before recovery was ascertained, and one patient never recovered through the 10-year follow-up. Among 84 patients who once recovered, 10 never presented with a full relapse, 29 experienced a full relapse, 11 experienced a subthreshold relapse, and 33 were lost to follow-up without ever recording full relapse.

In this naturalistic study, the cohort received, on average, 60 (SD = 44) mg of imipramine or equivalent per day on entry and 85 (SD = 73) mg at 1 month (Furukawa *et al.*, 2000b). During continuation phase, 62% (52/84) were prescribed no drugs at all. Among