

SLEEP DEPRIVATION REDUCES RAT HYPERHOMOCYSTEINEMIA INDUCED BY A HYPERLIPIDIC DIET

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ABSTRACT

Objective: Several clinical and experimental studies have shown an association between hyperhomocysteinemia and cardiovascular diseases (CVD). Long-term exposure to a hyperlipidic diet induces tissue fatty acid accumulation and increases circulating lipid concentrations, which raises the risk for CVD. Sleep debt is also considered to be an important factor enhancing cardiovascular risk. Since no information concerning homocysteine (Hcy) levels resulting from a hyperlipidic diet is currently available, our objective was to investigate changes in Hcy concentrations in sleep deprived rats fed a hyperlipidic diet. **Subjects and Methods:** Rats were maintained for 65 days on a high-fat diet, whereas control animals received regular food ad libitum. After this period, a sub-group of these animals was submitted to sleep deprivation (SD). Homocysteine, thiobarbituric acid reactive substances (TBARS), vitamin B6, folate, and the lipid profile were measured. **Results:** Hcy concentrations were significantly higher in hyperlipidic fed rats. Sleep deprivation reduces high levels of this amino acid as well as triacylglycerols and TBARS levels. **Conclusions:** The present study shows for the first time that plasma Hcy concentrations in rats increase as a result of a hyperlipidic diet consumption. Metabolic changes induced by SD interfered in CVD-related factors even after the use of a hyperlipidic diet.

Keywords: hyperhomocysteinemia; hyperlipidic diet; rats; sleep deprivation.

INTRODUCTION

Homocysteine (Hcy) is a sulfur-containing amino acid derived from methionine, and there are several possible metabolic pathways for its clearance from the system: remethylation to form

methionine via either cobalamin-dependent methionine synthase (using N5-methyltetrahydrofolate as a methyl donor) or betaine-homocysteine methyltransferase (using betaine as a methyl donor); catabolism through the transsulfuration pathway, ultimately forming cysteine; or export to the extracellular space (1,2). Hcy

metabolism and plasma concentration are regulated by vitamin B6, vitamin B12, and folic acid levels (2). An increasing number of current clinical and experimental studies have shown an association between deficiencies in dietary sources of vitamins (mainly folate and vitamin B12) and hyperhomocysteinemia (3).

Hyperhomocysteinemia is associated with cardiovascular, renal, and neurodegenerative diseases (2). Total plasma Hcy values of approximately 10 $\mu\text{mol/L}$ for men and 8 $\mu\text{mol/L}$ for women constitute the normal range (4). However, even a small increase in total plasma Hcy is associated with an increased risk of coronary artery disease both for men and women (5,6). Moreover, a number of studies have demonstrated that smoking, excessive alcohol consumption/alcohol abuse, obesity, type II diabetes, and an unhealthy diet contribute to mild hyperhomocysteinemia (2,4).

Long-term exposure to a hyperlipidic high fat diet induced marked tissue fatty acid accumulation and increased circulating lipid concentrations, which may influence or negatively impact cell function and contribute to the risk of cardiovascular disease (7). Moreover, a hyperlipidic diet may result in an increased heart rate and is generally believed to favor obesity and hypertension (8). Despite the great body of evidence relating Hcy to cardiovascular disease (and particularly associating Hcy with obesity and hypertension), no information is available concerning the causal relationship between a hyperlipidic diet and high Hcy levels. This is of particular interest since the significant change in current nutritional habits has been accompanied by an increased incidence of obesity and cardiovascular disease.

Furthermore, previous studies conducted by our group showed that sleep deprivation (SD) reduces Hcy levels in both young and aged rats (9,10). This unexpected effect was observed in regularly fed rats with Hcy levels within the normal range. We wondered if SD under hyperhomocysteinemic conditions would have the same effect. Therefore, our objective was to verify both the changes in total plasma Hcy concentration associated with a palatable hyperlipidic diet (HD) and the response to SD in rats.

METHODS AND MATERIALS

Animals

Male Wistar rats aged 21 days at the beginning of the experiment, which was conducted at the Department of Psychobiology from the Universidade Federal de São Paulo, were housed in standard polypropylene cages in a temperature-controlled ($23 \pm 1^\circ\text{C}$) room with a 12:12 h light-dark cycle (lights on at 07.00 am). All procedures adopted in the present study comply with the Guide for Care and Use of Laboratory Animals.

Each animal was weighed weekly during the experiment between 0800 and 0900 h. The animals were also weighed after the end of the SD procedure.

Hyperlipidic Diet

The experimental animals were maintained on a palatable, high-fat diet for 65 days, whereas control animals had access to regular food ad libitum. The hyperlipidic diet consisted of commercial rat chow plus peanuts, milk chocolate, and sweet biscuit in a proportion of 3:2:2:1 (11). All components were powdered

and mixed, and pellets were produced. The caloric density of the diet was determined with an adiabatic calorimeter (IKA-C400). The composition of both diets is shown in Table 1.

Table 1: Diet composition

	Hyperlipidic Diet (g/Kg)	Normal diet (g/Kg)
Proteins	190	190
Carbohydrates	470	560
Lipids	160	35
Cellulose	30	45
Minerals and vitamins	50	50
Total:	21.40 kJ/g	17.03 kJ/g

After 65 days on their respective diets, the animals were sacrificed by decapitation. Blood was collected in pre-cooled tubes containing ethylene diamine tetra acetic acid (EDTA) or heparin as an anticoagulant, and it was centrifuged at 900 g for 6 minutes at 4°C . Another tube free of anticoagulant was used to obtain samples for cholesterol and triacylglycerol analysis. Plasma and serum were extracted, transferred to microtubes, and stored at -80°C until biochemical assays were performed.

Another group of male Wistar rats was maintained on a high-fat diet for 65 days, whereas control animals were kept on regular feed ad libitum. After this period, a subgroup from each diet group was submitted to SD for 96 h using the platform technique. Control counterparts were allowed to sleep. During the SD procedure, all animals received a normal diet, because the feeder on the SD apparatus only permits this kind of food.

The final groups in the experiment were: normal diet (N = 8), hyperlipid diet (N = 8), sleep-deprived and normal diet (N = 8), and sleep-deprived and hyperlipidic diet (N = 8).

Plasma Hcy

Total plasma Hcy values were determined by high-performance liquid chromatography (HPLC) with fluorimetric detection and isocratic elution (12). This methodology involves three steps: the reduction of thiol groups using tris (carboxyethyl) phosphine, protein precipitation, and derivatization with 7-fluorobenzene-2-oxy-1,3-diazolic-4-ammonium sulfate (SBD-F). The HPLC system used was a Shimadzu apparatus with an SIL-10Dvp automatic sample injector and an RF-10AXL fluorescence detector. Chromatographic separation was performed using a Prodigy Phenomenex ODS2 column (3.2 mm x 150 mm, with $5\mu\text{m}$ microparticles). The fluorescence of the separated compounds was detected with a detector adjusted for excitation at 385 nm and emission at 515 nm. Total Hcy content was calculated with a calibration curve using known Hcy concentrations and cystamine as the internal standard. The intra-assay coefficients of variation (CV) for Hcy ranged from 1.1 to 1.8%, and the inter-assay CV was 5.6% (12).

Serum Lipids

Total cholesterol, HDL, LDL, VLDL, and triacylglycerol concentrations were assessed using colorimetric automatic procedures routinely performed in our clinical laboratory (Advia 16/50, Bayer

Diagnostics Corporation) using commercial kits (Dialab®).

Plasma folate and vitamin B6

Folate and vitamin B6 plasma concentrations were determined by HPLC analysis according to the method described by Sharma & Dakshinamurti (13). Samples were extracted using methaphosphoric acid (10%), and folate and vitamin B6 solutions were used as standards. The chromatographic procedure was performed using the same apparatus used for Hcy, but a SPD-10VP UV-VIS detector and a Phenomenex Bondclone C18 (10 µm, 300 x 3.9 mm) were used as well.

TBARS determination

Plasma lipid peroxidation was determined by the detection of thiobarbituric acid reactive substances (TBARS) using the methodology described by Ohkawa et al. (14). This methodology is based on the formation of a chromophoric compound after the reaction of malondyaldehyde with thiobarbituric acid spectrophotometrically measured at 535 nm.

Sleep Deprivation

The experimental group was submitted to SD using the modified multiple platform method. This involved placing the rats inside a tiled water tank (123 x 44 x 44 cm) containing 14 circular platforms, 6.5 cm in diameter, with the water level being 1 cm below their upper surface (15). The rats could thus move around inside the tank by jumping from one platform to another. When they reached the sleep paradoxical phase, muscle atonia commenced and they fell into the water, which woke them. Throughout the study, the experimental room was maintained under a controlled temperature (23 ± 1°C) with a 12:12 h light-dark cycle (lights on at 0700 am). Food and water were provided ad libitum; chow pellets and water bottles were placed on a grid located on the top of the tank. The water in the tank was changed daily throughout the SD period.

After the SD procedure, the animals were sacrificed by decapitation. Blood was collected for biochemical determinations as previously described.

Statistical analysis

The results for each variable were compared using a two-way ANOVA followed by the Tukey Test for pairwise comparisons (Statistica for Windows 1997, StatSoft, Inc., Tulsa, OK, USA). The level of significance was set at $p \leq 0.05$.

RESULTS

We found that rats maintained on a 65-day hyperlipidic diet showed higher volumes of fat deposits inside the abdominal cavity than controls regardless of SD. No significant increases in body weight were observed before the beginning of the SD period (416.8 ± 37.8 g vs. 413.3 ± 52.4 g for hyperlipidic diet vs. normal food, respectively). Rats from the sleep-deprived group exhibited lower body weight at the end of the SD period regardless of diet (normal food group: 414.4 ± 29.2 g vs. 379.4 ± 33.6 g and hyperli-

pidic diet group: 415.0 ± 30.7 g vs. 378.9 ± 27.1 g).

The analysis of total cholesterol levels revealed two main effects of group ($F(1,36)=18.26$; $p<0.0001$) and diet ($F(1,36)=26.82$; $p<0.0001$). Rats fed the hyperlipidic diet exhibited higher total cholesterol levels than those fed normal food (Table 2). Furthermore, sleep-deprived rats had higher cholesterol levels than controls. The same effects were present for LDL levels (group: $F(1,36)=46.85$; $p<0.0001$ and diet: $F(1,36)=15.46$; $p<0.001$) (Table 2).

Table 2: Biochemical parameters.

	ND Control	HD Control	ND Sleep deprived	HD Sleep deprived
Total Cholesterol (mg/dL)	57.9±7.4	67.7±7.9*	65.0±10.2#	86.1±11.6*#
LDL (mg/dL)	15.2±6.9	22.2±6.3*	36.7±6.4#	28.0±5.5*#
HDL (mg/dL)	25.3±2.7	27.3±4	27.8±5.2	37.7±6.5§
VLDL (mg/dL)	17.4±4.5	18.2±4.1	9.2±1.1#	11.7±1.7#
Triacylglycerol (mg/dL)	87.0±22.4	91,1±20.5	46.1±5.2#	58.7±8.4#
Homocysteine (µmol/L)	6.59±1.24	13.76±3.89*	5.59±0.88	7.22±1.72
Folate (µmol/L)	0.0198±0.009	0.0180±0.01	0.0216±0.009	0.0203±0.009
Vitamin B6 (mmol/L)	2.88±1.49	2.87±1.56	3.38±2.50	3.81±2.07
Lipid peroxidation (µmol TBARS)	2.48±0.79	1.78±1.17	0.38±0.51#	1.01±0.97#

Results are shown as mean ± standard deviation. ND – normal diet; HD – hyperlipidic diet. *Different from respective normal diet group. #Different from respective sleep deprived group. §Different from all other groups.

HDL cholesterol levels in sleep-deprived rats on a hyperlipidic diet were higher than those in all other groups studied (group vs. diet interaction $F(1,36)=6.75$; $p<0.01$; Table 2). Although the hyperlipidic diet did not induce hypertriacylglycerolemia, SD reduced both triacylglycerol and VLDL levels in both diet groups ($F(1,36)=52.73$; $p<0.0001$) (Table 2).

Sleep deprivation reduces TBARS levels independent of diet (group vs. diet interaction: $F(1,30)=4.51$; $p<0.05$; Table 2). Homocysteine concentrations were significantly higher in rats on hyperlipidic than normal diets (Mean 13.75 µmol/L vs. 6.59 µmol/L; group vs. diet interaction: $F(1,36)=15.03$; $p<0.001$;

Table 2). Curiously, 96 hours of SD significantly reduced Hcy levels and brought those of hyperhomocysteinemic rats to normal concentrations (13.75 $\mu\text{mol/L}$ to 7.21 $\mu\text{mol/L}$).

No differences in folate or vitamin B6 were observed between groups ($p = 0.86$ and $p = 0.24$, respectively).

DISCUSSION

Consumption of a palatable hyperlipidic diet by rats for 65 days produced hyperhomocysteinemia. This finding is of great interest since Western people are consuming increasing amounts of lipids and carbohydrates as part of their regular diet and the incidence of cardiovascular problems is rising every year. Higher Hcy levels induced by hyperlipidic diets have been previously described in humans who showed a positive correlation between Hcy and the consumption of fat and calories (16). This is, however, the first description of hyperhomocysteinemia in rats as a consequence of this kind of diet.

Mild hyperhomocysteinemia in rats usually results from methionine-rich or folate-deficient diets (17) that affect Hcy metabolism by up-regulating the conversion of Hcy back to methionine or cysteine. It is difficult to explain how a hyperlipidic diet leads to higher Hcy concentrations, since neither folate levels (the peanuts added to the diet provided an extra 145 μg of folic acid per 100 g of peanuts) nor methionine content changed with this kind of diet. In fact, folate concentrations in hyperlipidic-diet rats were very similar to those of normally fed animals; the same was true of vitamin B6 levels (Table 2). Taken together, these results suggest that hyperhomocysteinemia was not the result of mechanisms related to the impairment of remethylation or transsulfuration pathways due to changes in the availability of vitamins.

High fat diets impair glucose metabolism, stimulate abnormal glucose production, and lead to hyperinsulinemia and insulin resistance (18). Cholesterol levels are also affected by high fat diets (11,19). Hyperhomocysteinemia has been associated with abnormal glucose and cholesterol metabolism in both humans (20-22) and animals (23,24). Although there are no apparent causal relationships between these findings, the hyperlipidic diet rats in our study that presented hyperhomocysteinemia also showed high levels of total cholesterol and LDL.

It is interesting that data from the Hordaland Homocysteine Study suggest that high saturated fat intake in humans is associated with higher total Hcy concentrations (25). In that cross-sectional, population-based study of 5917 subjects in two age groups (47-19 and 71-74 years old), an approximately 15 gram increase in saturated fat intake was associated with a 6% increase in Hcy concentrations after adjustment for age, sex, energy intake, and other factors. In that study, this association is of the same magnitude as that predicted between saturated fat intake and LDL cholesterol (26).

Homocysteine concentrations in sleep-deprived rats maintained on normal food were lower but not statistically significantly different from those of control rats. However, previous work conducted by our group showed lower concentrations of Hcy levels after SD (10,27). In fact, the higher values presented by hyperlipidic-diet rats interfered with the distribution of the val-

ues obtained for the other groups and thereby precluded statistical significance. In light of this, no statistically significant difference between hyperlipidic- and control-diet rats could be observed. Interestingly, however, SD reduced Hcy levels to normal values in rats with high Hcy concentrations.

Notably, some parameters related to lipid metabolism either did not change after SD or changed in the opposite direction. For example, SD produced an increase in both total and LDL cholesterol. HDL cholesterol levels did not change after SD in animals that received the normal diet, but they were higher in HD sleep-deprived animals.

Since SD produces important metabolic alterations related to increased risk for cardiovascular disease (28-31), it would be interesting to establish the mechanisms that decrease Hcy concentrations in rats. It is important to emphasize that this decrease brought Hcy concentrations to normal values despite the fact that animals were maintained on a hyperlipidic diet. Reduction in Hcy concentrations is considered a valuable tool for decreasing cardiovascular risk in humans (32-35). Because of this, the decrease in Hcy concentrations associated with SD in rats warrants further investigation.

SD in humans has been associated with altered Hcy concentrations (36,37). In these studies, however, changes occurred in opposite directions. Sleep-deprived shift workers had higher Hcy concentrations than regular-schedule controls (18 vs 8 $\mu\text{mol/L}$). Patients suffering from sleep apnea also presented high Hcy concentrations (37-41). These patients were submitted to several episodes of hypoxia/re-oxygenation, which may explain their Hcy augmentation (37). For the shift workers, changes in feeding patterns may be related to both hyperhomocysteinemia and changes in other cardiovascular risk factors. All parameters analyzed showed variations associated with increased cardiovascular risk (36).

Sleep deprivation reduced the levels of TBARS in both groups. This finding was already observed by our group (27), and it is probably related to difficulty in reaching the necessary amount of food during the SD period (42). Some authors have shown that caloric restriction reduces oxidative stress; in this sense, the hyperlipidic diet did not prevent SD from lowering levels of TBARS, besides being less effective.

The present study shows that some CV risk factors in rats were reduced after SD even when rats were fed a hyperlipidic diet. The reason for this reduction remains to be elucidated. We believe that the higher energy intake induced by SD may produce a metabolic status characterized by hypermethylation, which in turn, may decrease Hcy availability.

The regulation of the genetic expression is tightly controlled and well balanced in the body by different epigenetic mechanisms, such as DNA methylation and histone modification. DNA methylation occurring after embryogenesis is seen primarily as an irreversible event, and even small changes in genomic DNA methylation may have biological relevance. Several factors influencing DNA methylation, including Hcy, have already been identified.

Sleep deprivation is considered to produce a hypermetabolic status. Cirelli and co-workers analyzed the expression of more than 26,000 transcripts in the cerebral cortex after SD in rats (43) and found an increase in the expression of 75 genes. Among these were genes related to metabolism, the immune response, and stress. Since

Hcy is a product of the methylation pathway, changes in its levels may be considered a biological marker of gene expression.

This may, in turn, suggest molecular changes induced by SD.

In conclusion, rats fed a hyperlipidic diet showed increased plasma Hcy concentrations that were decreased after sleep deprivation. Continued research on the role of methylation reactions during SD will lead to greater understanding of the possible mechanisms responsible for these findings.

REFERENCES

- Jacobsen D.W. Homocysteine and vitamins in cardiovascular disease. *Clin Chem* 1998; 44: 1833-1843.
- Selhub J. Homocysteine metabolism. *Annual Reviews of Nutrition* 1999; 19: 217-246.
- Motulsky AG. Nutritional ecogenetics: homocysteine-related arteriosclerotic vascular disease, neural tube defects, and folic acid. *American Journal of Human Genetics* 1996; 58: 17-20.
- Refsum H, Smith AD, Ueland PM, Nexø E, Clarke R, McPartlin J, Johnston C, Engbaek F, Schneede J, McPartlin C, Scott JM. Facts and recommendations about total homocysteine determinations: an expert opinion. *Clinical Chemistry* 2004; 50: 3-32.
- Boushey CJ, Beresford SAA, Omenn GS, Motulsky AG. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes. *JAMA* 1995; 274: 1049-1057.
- Tavares JR, D'Almeida V, Diniz DC, Terzi CA, Cruz EN, Stefanini E, Andriollo, A, Paola AA, Carvalho AC. Analysis of plasma homocysteine levels in patients with unstable angina. *Arquivos Brasileiros de Cardiologia* 2002; 79: 161-172.
- Hill JO, Fried SK, DiGirolamo M. Effects of a high-fat diet on energy intake and expenditure in rats. *Life Sci* 1983; 33: 141-149.
- Corbett SW, Stern JS, Keesey RE. Energy expenditure in rats with diet-induced obesity. *Am. J. Clin. Nutr* 1986; 44: 173-180.
- Oliveira AC, D'Almeida V, Hipólido DC, Nobrega JN, Tufik S. Sleep deprivation reduces total plasma homocysteine levels in rats. *Canadian Journal of Physiology and Pharmacology* 2002; 80: 193-197.
- Andersen ML, Martins PJF, D'Almeida V, Santos RF, Bignotto M, Tufik S. Effects of paradoxical sleep deprivation on blood parameters associated with cardiovascular risk in aged rats. *Experimental Gerontol* 2004; 39: 817-824.
- Estadella D, Oyama LM, Damaso AR, Ribeiro EB, Oller Do Nascimento CM. Effect of palatable hyperlipidic diet on lipid metabolism of sedentary and exercised rats. *Nutrition* 2004; 20: 218-224.
- Pfeiffer CM, Huff DL, Gunter EW. Rapid and accurate HPLC assay for plasma total homocysteine and cysteine in a clinical laboratory setting. *Clinical Chemistry* 1999; 45: 290-292.
- Sharma SK, Dakshinamurti K. Determination of vitamin B6 vitamers and pyridoxic acid in biological samples. *Journal of Chromatography* 1982; 578: 45-51.
- Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry* 1979; 95: 351-358.
- Nunes Junior GP, Tufik S, Nobrega JN. Decreased muscarinic receptor binding in rat brain after paradoxical sleep deprivation: an autoradiographic study. *Brain Research* 1994; 645: 247-252.
- Poirier LA, Wise CK, Delongchamp RR, Sinha R. Blood determinations of S-adenosylmethionine, S-adenosylhomocysteine, and homocysteine: correlations with diet. *Cancer Epidemiology Biomarkers & Prevention* 2001; 10: 649-655.
- Zhang R, Ma J, Xia M, Zhu H, Ling W. Mild hyperhomocysteinemia induced by feeding rats diets rich in methionine or deficient in folate promotes early atherosclerotic inflammatory processes. *Journal of Nutrition* 2004; 134: 825-830.
- Akiyama T, Tachibana I, Shirohara H, Watanabe N, Otsuki M. High-fat hypercaloric diet induces obesity, glucose intolerance and hyperlipidemia in normal adult male Wistar rat. *Diabetes Res. Clin. Pract* 1996; 31: 27-35.
- Marotta M, Ferrer-Martnez A, Parnau J, Turini M, Mace K, Gomez Foix AM. Fiber type- and fatty acid composition-dependent effects of high-fat diets on rat muscle triacylglyceride and fatty acid transporter protein-1 content. *Metabolism* 2004; 53: 1032-1036.
- Setola E, Monti LD, Galluccio E, Palloschi A, Fragasso G, Paroni R, et al. Insulin resistance and endothelial function are improved after folate and vitamin B12 therapy in patients with metabolic syndrome: relationship between homocysteine levels and hyperinsulinemia. *European Journal of Endocrinology* 2004; 151, 483-489.
- Ajabnoor MA, AL-Ama MN, Banjar Z, Rafee AA, Sheweita SA. Homocysteine level and other biochemical parameters in cardiovascular disease patients with diabetes mellitus. *Med. Sci. Monit* 2003; 9: 523-527.
- Oron-Herman M, Rosenthal T, Sela BA. Hyperhomocysteinemia as a component of syndrome X. *Metabolism* 2003; 52: 1491-1495.
- Namekata K, Enokido Y, Ishii I, Nagai Y, Harada T, Kimura H. Abnormal lipid metabolism in cystathionine beta-synthase-deficient mice, an animal model for hyperhomocysteinemia. *Journal of Biology Chemistry* 2004; 279: 52961-52969.
- Zulli A, Hare DL, Buxton BF, Black MJ. High dietary methionine plus cholesterol exacerbates atherosclerosis formation in the left main coronary artery of rabbits. *Atherosclerosis* 2001; 176: 83-89.
- Berstad P, Konstantinova SV, Refsum H, Nurk E, Vollset SE, Tell GS, et al. Dietary fat and plasma total homocysteine concentrations in 2 adult age groups: the Hordaland Homocysteine Study. *Am J Clin Nutr* 2007; 85:1598-1605.
- Verhoef P. Homocysteine - an indicator of a healthy diet? *Am J Clin Nutr* 2007; 85: 1446-1447.
- de Oliveira AC, D'Almeida V, Hipólido DC, Nobrega JN, Tufik S. Sleep deprivation reduces total plasma homocysteine levels in rats. *Can J Physiol Pharmacol* 2002; 80: 193-197.
- Meier-Ewert HK, Ridker PM, Rifai N, Regan MM, Price NJ, Dinges DF, et al. Effect of sleep loss on C-reactive protein, an inflammatory marker of cardiovascular risk. *Journal of American College of Cardiology* 2004; 43: 678-683.
- Shamsuzzaman AS, Caples SM, Somers VK. Sleep deprivation and circulatory control. *Sleep* 2003; 26: 934-936.
- Boethel CD. Sleep and the endocrine system: new associations to old diseases. *Curr. Opin. Pulm. Med* 2002; 8: 502-505.
- Palma BD, Gabriel AJr, Bignotto M, Tufik S. Paradoxical sleep deprivation increases plasma endothelin levels. *Brazilian Journal of Medical and Biological Research* 2002; 35: 75-79.
- Vincent S, Gerber M, Bernard MC, Defoort C, Loundou A, Portugal H, et al. The Medi-RIVAGE study (Mediterranean Diet, Cardiovascular Risks and Gene Polymorphisms): rationale, recruitment, design, dietary intervention and baseline characteristics of participants. *Public Health*

Nutrition 2004; 7: 531-542.

33. McCully KS. Homocysteine, vitamins, and prevention of vascular disease. *Military Medicine* 2004; 169: 325-329.
34. Hackam DG, Anand SS. Emerging risk factors for atherosclerotic vascular disease: a critical review of the evidence. *JAMA* 2003; 290: 932-940.
35. Das UN. Folic acid says NO to vascular diseases. *Nutrition* 2003; 19: 686-692.
36. Martins PJ, D'Almeida V, Vergani N, Perez AB, Tufik S. Increased plasma homocysteine levels in shift working bus drivers. *Occup Environ Med* 2003; 60: 662-666.
37. Lavie L, Perelman A, Lavie P. Plasma homocysteine levels in obstructive sleep apnea: association with cardiovascular morbidity. *Chest* 2001; 120: 900-908.
38. Robinson GV, Pepperell JC, Segal HC, Davies RJ, Stradling JR. Circulating cardiovascular risk factors in obstructive sleep apnoea: data from randomised controlled trials. *Thorax* 2004; 59: 777-782.
39. Svatikova A, Wolk R, Magera MJ, Shamsuzzaman AS, Phillips BG, Somers VK. Plasma homocysteine in obstructive sleep apnoea. *European Heart Journal* 2004; 25: 1325-1329.
40. Winnicki M, Palatini P. Obstructive sleep apnoea and plasma homocysteine: an overview. *European Heart Journal* 2004; 25: 1281-1283.
41. Phillips BG, Somers VK. Sleep disordered breathing and risk factors for cardiovascular disease. *Current Opinion in Pulmonary Medicine* 2002; 8: 516-520.
42. Martins PJ, D'Almeida V, Nobrega JN, Tufik S. A reassessment of the hyperphagia/weight-loss paradox during sleep deprivation. *Sleep* 2006; 29: 1233-1238.
43. Cirelli C, Faraguna U, Tononi G. Changes in brain gene expression after long-term sleep deprivation. *J Neurochem* 2006; 98: 1632-1645.