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Melatonin stimulates proliferation and type I collagen synthesis in human bone cells in vitro

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Abstract: The pineal secretory product melatonin reportedly regulates release of growth hormone in humans and prevents phototherapy-induced hypocalcemia in newborn rats, suggesting that melatonin affects bone metabolism. Little is known about the effects of melatonin on bone in vitro or in vivo. The present study was undertaken to examine whether melatonin acts directly on normal human bone cells (HOB-M cells) and human osteoblastic cell line (SV-HFO cells) to affect osteogenic action in vitro. The effect of melatonin on bone cell proliferation was determined using the 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT) assay after a 24 hr incubation with melatonin. Melatonin significantly and dose-dependently increased the proliferation in HOB-M cells and SV-HFO cells by 215 ± 22.1%, and 193 ± 6.4%, respectively, with a maximal effect at a concentration of 50 μM. To evaluate the effect of melatonin on bone cell differentiation, alkaline phosphatase (ALP) activity, osteocalcin secretion and procollagen type I c-peptide (PICP) production (a measure of type I collagen synthesis) were measured after a 48 hr treatment. While melatonin at micromolar concentrations did not significantly affect either the ALP activity or the osteocalcin secretion, it significantly and dose-dependently increased the PICP production in HOB-M cells and SV-HFO cells by 983 ± 42.2%, and 139 ± 4.2%, respectively, with the maximal stimulatory doses between 50 and 100 μM. These results provide new evidence that melatonin stimulates the proliferation and type I collagen synthesis in human bone cells in vitro, suggesting that melatonin may act to stimulate bone formation.

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Introduction

Melatonin, a secretory product synthesized nocturnally by the pineal gland, plays a major role in the coordination of seasonal reproduction [Reiter, 1991] and pubertal development in some mammals [Silman et al., 1979]. Melatonin is a potent antioxidant agent [Marshall et al., 1996; Reiter et al., 1997] and may possess anti-aging [Sandyk, 1990; Armstrong and Redman, 1991] and oncostatic properties [Blask et al., 1988; Lissoni et al., 1994; Kojima et al., 1997; Panzer and Viljoen, 1997]. Melatonin also influences release of growth hormone in humans [Smythe and Lazarus, 1974; Valcavi et al., 1993], serum levels of corticosterone in rats [Oxenkrug et al., 1984] and the circadian rhythm of osteoblast metabolism in rats [Cane et al., 1991]. In addition, melatonin prevents phototherapy-induced hypocalcemia in newborn rats [Hakanson and Bergsrom, 1981]. These findings suggest that melatonin affects bone and mineral metabolism. Furthermore, considerable indirect evidence links melatonin to osteoporosis [Sandyk et al., 1992]. For example, melatonin secretion decreases sharply during menopause [Sack et al., 1986], declines with immobility [Vaughan et al., 1978; Yocca and Freidman, 1984] and increases with exercise [Carr et al., 1981]. Osteoporosis and pineal calcification are uncommon among black populations [Adeloye and Felson, 1974]. While melatonin inhibited proliferation of human breast cancer cells [Hill and Blask, 1988; Molis et al., 1994], human benign prostate epithelial cells [Gilad et al., 1997] and rat hepatocytes [Kojima et al., 1997], its effect on bone cells have not been

addressed. The present study was to examine whether melatonin affects proliferation and differentiation in human bone cells in vitro. In this report, we provide new evidence that melatonin at micromolar concentrations stimulates proliferation and type I collagen synthesis in human bone cells in vitro.

Materials and methods

Materials

Tissue culture supplies were obtained from Iwaki Glass (Funabashi, Japan) or Falcon (Oxnard, CA). Dulbecco's modified Eagle's medium (DMEM), 0.5% trypsin–5.3 mM EDTA solution and collagenase were products of GIBCO/BRL Life Technologies (Grand Island, NY). Iron-supplemented bovine calf serum was purchased from JRH Biosciences (Lenexa, KS). Melatonin, bovine serum albumin (BSA), 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT), Triton X-100, dimethyl sulfoxide (DMSO) and p-nitrophenyl phosphate (pNPP) were products of Sigma Chemical Co. (St. Louis, MO). 1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) was obtained from Biomol Research (Plymouth Meeting, PA). Gla-type osteocalcin EIA and procollagen type I c-peptide (PICP) EIA kits were purchased from Takara (Ohtsu, Japan). All other chemicals were reagent grade, either from Sigma Chemical Co. or from Kanto Chemical Co. (Tokyo, Japan).

Cell cultures

Normal human bone cells were isolated from mandibular bone samples by collagenase digestion as previously described [Wegedal and Baylink, 1984]. The resulting bone cells (HOB-M cells) were shown to be of osteogenic nature, based on their responsiveness to parathyroid hormone (PTH) for increased cAMP production and to $1,25(OH)_2D_3$ for increased alkaline phosphatase (ALP) activity and osteocalcin secretion [Wegedal and Baylink, 1984; Nakade et al., 1995]. The cells were maintained in DMEM supplemented with 10% bovine calf serum and passaged every week at a 1:4 dilution ratio. Cells from passages 3–7 were examined in this study. Simian virus 40-immortalized human osteoblastic cell line (SV-HFO cells) were kindly provided by Dr. Hideki Chiba (Department of Pathology, Sapporo Medical University, Sapporo, Japan). Two kinds of human bone cells (HOB-M cells and HFO cells) were respectively plated at a density of 7,500 cells/cm^2 (for proliferation assay) or 10,000 cells/cm^2 (for ALP, osteocalcin and PICP assays) in DMEM supplemented with 10% bovine calf serum. After the cells attached to the plates for 24 hr, the culture medium was changed to fresh serum-free DMEM-containing 0.01% BSA. Twenty-four hours later, effectors (e.g., melatonin) were added, and the cells were incubated for either 24 hr for the proliferation assay or 48 hr for ALP, osteocalcin and PICP assays. Melatonin stock solutions were dissolved in DMSO and diluted in DMEM-containing 0.01% BSA immediately before use. The final DMSO concentration was 0.1%. The same DMSO concentration was included in each assay as vehicle control.

Effect on cell proliferation

The effect of melatonin on the bone cell proliferation was assessed by XTT assay [Roehm et al., 1991], after a 24 hr incubation with melatonin in serum-free DMEM containing 0.01% BSA. The assay is dependent on a cellular reduction of XTT by the mitochondrial dehydrogenase of viable cells to a red formazan product which represents the cell proliferative activity [Roehm et al., 1991]. The mitogenic effect was compared with that of ascorbic acid which is also an antioxidant agent [Glascott et al., 1996]. Each treatment group consisted of six replicates.

Effect on cell differentiation

Cellular ALP activity, osteocalcin and type I collagen are generally regarded as osteoblastic differentiation markers [Farley and Baylink, 1986; Owen et al., 1990; Pockwinse et al., 1992; Termine and Robey, 1996]. Thus, we evaluated the effect of malatonin on differentiation of human bone cells by monitoring cellular ALP activity, osteocalcin secretion and type I collagen synthesis. Cellular ALP activity was assayed as the rate of hydrolysis of pNPP at pH 10.3 per mg protein [Farley and Jorch, 1983]. Cell extracts were prepared with 0.1% Triton X-100 and the enzyme activity was normalized against cellular protein [Wiechelman et al., 1988]. Osteocalcin secretion in conditioned medium was assayed using a specific Gla-type osteocalcin EIA kit after a 48 hr treatment [Koyama et al., 1991]. The effect was tested in the presence of 10^{-8} M $1,25\ (OH)_2D_3$, because both HOB-M cells and SV-HFO cells did not secrete detectable amounts of osteocalcin under basal conditions [Nakade et al., 1995]. The amount of osteocalcin in the conditioned medium was normalized against cellular protein content. PICP is a cleaved peptide before fibril formation during the

extracellular processing of type I collagen and represents cellular type I collagen synthesis. Thus, the effect on the type I collagen synthesis was indirectly assessed by PICP production in conditioned medium using a specific EIA kit after a 48 hr treatment [Suzuki et al., 1995]. The amount of PICP in the conditioned medium was normalized against cell number [Nakade et al., 1995].

Statistical methods

Results are shown as mean ± S.E.M. (n = 6). Statistical significance of the difference from the vehicle controls was analyzed with a two tailed Student's t-test. Statistical significance of the difference between the groups was determined by one-way analysis of variance (ANOVA). The difference was considered significant when $P < 0.05$.

Results

Stimulation of the proliferation of human bone cells by melatonin in vitro

Fig. 1 shows that melatonin at micromolar concentrations significantly and dose-dependently increased the proliferation in HOB-M cells (215 ± 9.0%, $P < 0.001$ by ANOVA) and SV-HFO cells (192 ± 6.4%, $P < 0.005$ by ANOVA), with a maximal effect at 50 μM. In the same experiment, 10–50 μM ascorbic acid failed to significantly influence it in either HOB-M or SV-HFO cells (data not shown). These findings indicate melatonin at micromolar concentrations can act as a mitogen in human bone cells in vitro.

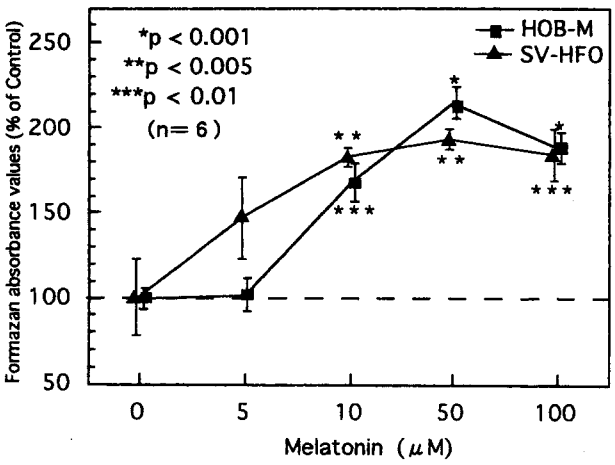

Fig. 1. Effect of melatonin on cell proliferation in human bone cells in vitro. Formazan absorbance values reflect proliferative activity of the cells in the XTT assay. Melatonin at micromolar concentrations significantly stimulated the proliferation in both HOB-M cells (215 ± 9.0%, $P < 0.001$ by ANOVA) and SV-HFO cells (192 ± 6.4%, $P < 0.005$ by ANOVA), in a dose-dependent manner with an optimal concentration of 50 μM. Results are shown as relative percentage (mean ± S.E.M.) of vehicle-treated control (n = 6 for each group). Dotted line represents 100% of vehicle control.

Table 1. Effect of mitogenic concentrations of melatonin on ALP activity in human bone cells in vitro

	ALP activity (μU/mg protein ± S.E.M.)	
Melatonin (μM)	HOB-M	SV-HFO
0 (vehicle control)	14.7 ± 0.7	3,618 ± 263
5	12.9 ± 0.5§	3,854 ± 420§
10	14.5 ± 1.2§	3,426 ± 318§
50	12.4 ± 0.8§	4,002 ± 257§
100	12.5 ± 0.8§	3,595 ± 347§

Cellular ALP activity was determined after a 48 hr treatment of mitogenic doses of melatonin. Enzyme activity was normalized against cellular protein. Melatonin treatment did not significantly influence the ALP activity in either HOB-M nor SV-HFO cells (by ANOVA). Results are shown as mean ± S.E.M. (n = 6 for each group).
§ Not significant compared with vehicle control by Student's t-test.

Effect of melatonin on differentiation of normal human bone cells in vitro

As shown in Tables 1 and 2, melatonin at micromolar concentrations failed to significantly affect either the ALP activity or the osteocalcin secretion in HOB-M and SV-HFO cells. On the other hand, as shown in Table 3, melatonin at micromolar concentrations significantly and dose-dependently

Table 2. Effect of melatonin on osteocalcin secretion of human bone cells

	Osteocalcin levels (ng/mg protein ± S.E.M.)	
Melatonin (μM)	HOB-M	SV-HFO
0 (vehicle control)	11.1 ± 1.2	17.6 ± 1.8
5	13.3 ± 0.9§	17.7 ± 2.3§
10	13.5 ± 0.6§	19.1 ± 2.6§
50	14.4 ± 0.9§	19.0 ± 2.6§
100	12.1 ± 2.1§	17.2 ± 2.4§

The cells were treated with 0–100 μM of melatonin in the presence of 10^{-8} M of $1,25(OH)_2D_3$ for 48 hr. The amount of osteocalcin in the conditioned media was normalized against cellular protein content. Melatonin did not significantly affect the osteocalcin secretion in either HOB-M or SV-HFO cells (by ANOVA). Results are shown as mean ± S.E.M (n = 6 for each group).
§ = Not significant compared with vehicle control by Student's t-test.

Table 3. Effect of melatonin on type I collagen synthesis in human bone cells

Melatonin (μM)	PICP levels (ng/10,000 cells ± S.E.M.) HOB-M (% control)	SV-HFO (% control)
0 (vehicle control)	31.4 ± 4.2 (—)	187 ± 13.3 (—)
5	70.2 ± 5.7 (223 ± 18.1)*	235 ± 6.3 (125 ± 3.4)**
10	71.9 ± 7.6 (229 ± 24.2)*	234 ± 5.1 (125 ± 2.7)**
50	309 ± 13.3 (983 ± 42.2)*	249 ± 7.0 (133 ± 3.7)**
100	192 ± 6.4 (612 ± 20.4)*	260 ± 7.8 (139 ± 4.2)*

The effect on type I collagen synthesis was indirectly estimated by the amount of PICP in conditioned medium using a specific EIA kit, after a 48 hr treatment. The amount of PICP was normalized against cell number. Melatonin at micromolar concentrations significantly increased the PICP production, in either HOB-M cells ($P < 0.001$ by ANOVA) or SV-HFO cells ($P < 0.001$ by ANOVA). Results are shown as mean ± S.E.M (n = 6 for each group).
* $P < 0.001$, ** $P < 0.005$, *** $P < 0.01$ compared with vehicle control by Student's t-test.

increased the PICP production in either HOB-M or SV-HFO cells by 983 ± 42.2% ($P < 0.001$ by ANOVA) and 139 ± 4.2% ($P < 0.001$ by ANOVA), respectively, with the maximal stimulatory doses between 50 and 100 μM. These findings indicate that mitogenic concentrations of melatonin can stimulate type I collagen synthesis, but neither ALP activity nor osteocalcin secretion in human bone cells.

Discussion

The present study demonstrates that melatonin at micromolar concentrations stimulates the proliferation and type I collagen synthesis in human bone cells (HOB-M cells) and human osteoblastic cell line (SV-HFO cells) in vitro. Although melatonin significantly increased the type I collagen synthesis in both human osteoblastic cells, the magnitude of the stimulation was different between HOB-M cells (983 ± 42.2%) and SV-HFO cells (139 ± 4.2%), indicating that it varies depending on cell type. The concentrations of melatonin that stimulated proliferation and type I collagen synthesis (from 10^{-5} to 10^{-4} M) in human bone cells in the study are higher than either circulating serum (from 10^{-11} to 10^{-9} M) or pharmacological levels (from 10^{-7} to 10^{-5} M) applied to breast cancers [Vaughan et al., 1978; Panzer and Viljoen, 1997], preliminarily suggesting that our in vitro observations may not be clinically relevant. However, our recent preliminary work suggested that the daily injection of melatonin at 5–50 mg/kg/day increased the tibial bone mineral density and bone volume in both growing young mice and ovariectomized old rats (unpublished data). Thus, the observed in vitro osteogenic effects of melatonin may be clinically relevant, and melatonin might be a potential useful therapeutic agents in situations where increased bone formation is desired such as fracture healing and osteoporosis.

Little is known about the mechanisms regarding the mitogenic action of melatonin on bone cells, whereas melatonin exerts the inhibitory effect on the proliferation of human breast cancer cells through regulation of the estrogen receptor [Molis et al., 1994]. It is also unclear why melatonin selectively increases type I collagen synthesis in the three kinds of osteoblastic differentiation markers. In this regard, the gene expression of type I collagen peaks earlier, compared with that of ALP or osteocalcin during osteoblastic maturation [Owen et al., 1990; Pockwinse et al., 1992]. Therefore, we suppose that melatonin may act on bone cells to stimulate the proliferation and relatively early stage of the differentiation. Further studies are, however, needed to elucidate it.

In conclusion, we provide new evidence in this paper that melatonin stimulates proliferation and type I collagen synthesis of human bone cells in vitro, suggesting that melatonin may act as a potent stimulator of bone formation.

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Literature cited

ADELOYE, A., B. FELSON (1974) Incidence of normal pineal gland calcification in skull roentgenograms of black and white Americans. Am. J. Radiol. 122:503–507.

ARMSTRONG, S.M., J.R. REDMAN (1991) Melatonin: A chronobiotic with anti-aging properties? Med. Hypotheses 34:300–309.

BLASK, D.E., S.M. HILL, D.B. PELLETIER (1988) Oncostatic signaling by the pineal gland and melatonin in the control of breast cancer. In: The Pineal Gland and Cancer, D. Gupta, A Attanasio, R.J. Reiter, eds. Brain Research Promotion, London, pp. 195–206.

CANE, V., D. FARNETI, G. MAROTTI, M. CRISCUOLO, G.P. TRENTINI, F. MAROTTI (1991) The effect of melatonin on bone growth in rats kept under constant light. Calcif. Tiss. Int.: A 47 (abstract).

CARR, D.D.B., S.M. REPPERT, B. BULLEN, G. SKRINAR, I. BEITINS, M. ARNOLD, M. ROSENBLATT, J.B. MARTIN, J.W. MCARTHUR (1981) Plasma melatonin increases during exercise in women. J. Clin. Endocrinol. Metab. 53:224–225.

FARLEY, J.R., U.M. JORCH (1983) Differential effects of phospholipids on skeletal alkaline phosphatase activity in extracts, in situ, and circulation. Arch. Biochem. Biophys. 221:477–488.

FARLEY, J.R., D.J. BAYLINK (1986) Skeletal alkaline phosphatase activity as a bone formation index in vitro. Metabolism 35:563–571.

GILAD, E., H. MATZKIN, N. ZISAPEL (1997) Interplay between sex steroids and melatonin in regulation of human benign prostate epithelial cell growth. J. Clin. Endocrinol. Metab. 82:2535–2541.

GLASCOTT, P.A. Jr, M. TSYGANSKAYA, E. GILFOR, M.A. ZERN, J.L. FARBER (1996) The antioxidant function of the physiological content of vitamin C. Mol. Pharmacol. 50:994–999.

HAKANSON, D.O., W.H. BERGSROM (1981) Phototherapy-induced hypocalcemia in newborn rats: Prevention by melatonin. Science 214:807–809.

HILL, S.M., D.E. BLASK (1988) Effects of the pineal hormone melatonin on the proliferation and morphological characteristics of human breast cancer cells (MCF-7) in culture. Cancer Res. 48:6121–6126.

KOJIMA, T., C. MOCHIZUKI, T. MITAKA, Y. MOCHIZUKI (1997) Effects of melatonin on proliferation, oxidative stress and Cx32 gap junction protein expression in primary cultures of adult rat hepatocytes. Cell Struct. Funct. 22:347–356.

KOYAMA, N., K. OHARA, H. YOKOTA, T. KUROME, M. KATAYAMA, F. HINO, I. KATO, T. AKAI (1991) A one step sandwich enzyme immunoassay for γ-carboxylated osteocalcin using monoclonal antibodies. J. Immunol. Methods 139:17–23.

LISSONI, P., S. BARNI, A. ARDIZZOIA, G. TANCINI, A. CONTI, G. MAESTRONI (1994) A randomized study with the pineal hormone melatonin versus supportive care alone in patients with brain metastases due to solid neoplasms. Cancer 73:699–701.

MARSHALL, K.A., R.J. REITER, B. POEGGELER, O.I. ARUOMA, B. HALLIWELL (1996) Evaluation of the antioxidant activity of melatonin in vitro. Free Rad. Biol. Med. 21:307–315.

MOLIS, T.M., L.L. SPRIGGS, S.M. HILL (1994) Modulation of estrogen receptor mRNA expression by melatonin in MCF-7 human breast cancer cells. Mol. Endocrinol. 8:1681–1690.

NAKADE, O., D.J. BAYLINK, K.-H.W. LAU (1995) Phenytoin at micromolar concentrations is an osteogenic agent for human-mandible-derived bone cells in vitro. J. Dent. Res. 74:331–337.

OWEN, T.A., M. ARONOW, V. SHALHOUB, L.M. BARONE, L. WILMING, M.S. TASSINARI, M.B. KENNEDY, S. POCKWINSE, J.B. LIAN, G.S. STEIN (1990) Progressive development of the rat osteoblast phenotype in vitro: Reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. J. Cell Physiol. 143:420–430.

OXENKRUG, G.F., I.M. MEINTYRE, S. GERSHON (1984) Effects of pinealectomy and aging on the serum corticosterone circadian rhythm in rats. J. Pineal Res. 1:181–185.

PANZER, A., M. VILJOEN (1997) The validity of melatonin as an oncostatic agent. J. Pineal Res. 22:184–202.

POCKWINSE, S.M., L.G. WILMING, D.M. CONLON, G.S. STEIN, J.B. LIAN (1992) Expression of cell growth and bone specific genes at single cell resolution during development of bone tissue-like organization in primary osteoblast. J. Cell Biochem. 49:310–323.

REITER, R.J. (1991) Pineal melatonin: Cell biology of its synthesis and of its physiological interaction. Endocr Rev. 12:151–180.

REITER, R.J., L. TANG, J.J. GARCIA, A. MUNOZ-HOYOS (1997) Pharmacological actions of melatonin in oxygen radical pathophysiology. Life Sci. 60:2255–2271.

ROEHM, N.W., G.H. RODGERS, S.M. HATFIELD, A.L. GLASEBROOK (1991) An improved colormetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT. J. Immunol. Methods 142:257–265.

SACK, R.L., A.J. LEWY, D.L. ERB, W.M. VOLLMER, C.M. SINGER (1986) Human melatonin production decreases with age. J. Pineal Res. 3:379–388.

SANDYK, R. (1990) Possible role of pineal melatonin in the mechanisms of aging. Int. J. Neurosci. 52:85–92.

SANDYK, R., P.G. ANASTASIADIS, P.A. ANNINOS, N. TSAGAS (1992) Is postmenopausal osteoporosis related to pineal gland functions? Int. J. Neurosci. 62:215–225.

SILMAN, R.E., R.M. LEONE, R.J.L. HOOPER, M.A. PREECE (1979) Melatonin, the pineal gland and human puberty. Nature 282:301–303.

SMYTHE, G.A., L. LAZARUS (1974) Growth hormone responses to melatonin. Science 184:1373–1374.

SUZUKI, R., M. KOBAYASHI, T. TAKIGUCHI, A. YAMAGUCHI, M. NAGUMO, K. Hasegawa (1995) Bone morphogenetic protein-2 enhances the expression of the osteoblast phenotypes in human periodontal ligament fibroblasts and osteoblasts. J. Bone Miner. Res. 10 (Suppl 1):T216 (abstract).

TERMINE, J.D., P.G. ROBEY (1996) Bone matrix proteins and the mineralization process. In: Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism, M. Favus, ed, 3rd. Lippincott-Raven, Philadelphia, pp. 24–28.

VALCAVI, R., M. ZINI, G.J. MAESTRONI, A. CONTI, I. PORTIOLI (1993) Melatonin stimulates growth hormone secretion through pathways other than the growth hormone-releasing hormone. Clin. Endocrinol. 39:193–199.

VAUGHAN, G.M., S.D. MCDONALD, R.M. JORDAN, J.P. ALLEN, G.L. BOHMFALK, M. ABOUSAMRA, J.L. STORY (1978) Melatonin concentration in human blood and cerebrospinal fluid: Relationship to stress. J. Clin. Endocrinol. Metab. 47:220–223.

WEGEDAL, J.E., D.J. BAYLINK (1984) Characterization of cells isolated and cultured from human bone. Proc. Soc. Exp. Biol. Med. 76:60–69.

WIECHELMAN, K., R. BRAUN, J. FITZPATRICK (1988) Investigation of bicinchoninin acid protein assay: Identification of the groups responsible for color formation. Anal. Biochem. 175:231–237.

YOCCA, F.D., E. FREIDMAN (1984) Effect of immobilization stress on rat pineal beta-adrenergic receptor-mediated function. J. Neurochem. 42:1417–1432.