Effect of combined lignan phytoestrogen and melatonin treatment on secretion of steroid hormones by adrenal carcinoma cells

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Objective—To investigate the in vitro effect of the combination of lignan enterolactone (ENL) or lignan enterodiol (END) with melatonin on steroid hormone secretion and cellular aromatase content in human adrenal carcinoma cells.

Sample—Human adrenocortical carcinoma cells.

Procedures—Melatonin plus ENL or END was added to cell culture medium along with cAMP ($100\mu M$); control cells received cAMP alone. Medium and cell lysates were collected after 24 and 48 hours of cultivation. Samples of medium were analyzed for progesterone, 17-hydroxyprogesterone, androstenedione, aldosterone, estradiol, and cortisol concentration by use of radioimmunoassays. Cell lysates were used for western blot analysis of aromatase content.

Results—The addition of ENL or END with melatonin to cAMP-stimulated cells (treated cells) resulted in significant decreases in estradiol, androstenedione, and cortisol concentrations at 24 and 48 hours, compared with concentrations in cells stimulated with cAMP alone (cAMP control cells). The addition of these compounds to cAMP-stimulated cells also resulted in higher progesterone and 17-hydroxyprogesterone concentrations than in cAMP control cells; aldosterone concentration was not affected by treatments. Compared with the content in cAMP control cells, aromatase content in treated cells was significantly lower.

Conclusions and Clinical Relevance—The combination of lignan and melatonin affected steroid hormone secretion by acting directly on adrenal tumor cells. Results supported the concept that this combination may yield similar effects on steroid hormone secretion by the adrenal glands in dogs with typical and atypical hyperadrenocorticism. (*Am J Vet Res* 2011;72:675–680)

The adrenal gland cortex secretes glucocorticoids, intermediate steroid hormones (sex steroid hormones), and mineralocorticoids. It is well known that clinical signs associated with hyperadrenocorticism in dogs result from excessive adrenal secretion of cortisol. What is not well known is that a variant of hyperadrenocorticism exists, referred to as atypical hyperadrenocorticism, in which cortisol concentration is within reference limits.[1] However, in animals with atypical disease, there is excessive secretion of adrenal intermediate steroid hormones,[1] some of which have been shown to induce clinical signs that mimic those caused by excess cortisol secretion.[1,2] Treatment of atypical hyperadrenocorticism with mitotane, a drug commonly used to treat dogs with naturally occurring hyperadrenocorticism,[3] is effective in lessening the secretion of most

adrenal intermediate steroid hormones, although it has a variable effect on estradiol secretion because sources of estradiol may include the adrenal glands,[4] gonads, and peripheral tissues such as adipose tissues and skin.[5]

A treatment that may lower concentrations of estradiol as well as concentrations of other intermediate steroid hormones is the combination of phytoestrogen[6–9] and melatonin.[10–15] It is our experience that the combination of lignan phytoestrogen and melatonin is effective in controlling atypical hyperadrenocorticism in some dogs. Phytoestrogens are plant-derived compounds with weak estrogenic activity and are classified as isoflavones, lignans, and coumestans. Lignans are found in whole grains, seeds, nuts, legumes, and vegetables; however, the most abundant source is flaxseed and, more specifically, flaxhulls.[16]

The main lignans in serum and urine of humans and other animals are the mammalian lignans ENL and END.[17] These compounds are referred to as mammalian lignans because they are formed by bacteria in the intestinal tract from the plant lignans matairesinol and

secoisolariciresinol.[17] Several in vitro studies[6–8] have revealed that treatment of certain cells with ENL and END decreases estrogen production and aromatase activity (an enzyme that converts androgens to estrogens). Furthermore, it has been suggested that lignans may affect uptake and metabolism of sex hormones by participating in the regulation of plasma sex hormone–binding globulin and may compete with estradiol for estrogen binding sites.[9]

Melatonin is a pineal gland hormone, the synthesis of which is influenced by photoperiod, with light suppressing its synthesis and darkness increasing its synthesis in mammals.[18] Melatonin has various physiologic functions,[18] including regulation of sexual reproduction in seasonal breeders.[10,11] Melatonin inhibits reproduction (antigonadotropic effect) during the winter months in long-day breeding animals[12] and reportedly inhibits ovarian activity in cats[10] and androgen production in isolated hamster Leydig cells.[11] In addition to decreasing sex hormone secretion, melatonin inhibits ACTH-stimulated cortisol production in primate adrenal glands[13] and modulates aromatase activity in various cell types.[14,15]

The purpose of the study reported here was to determine whether the combination of lignan and melatonin had an effect on adrenal steroid hormone secretion. Specifically, we sought to evaluate the effectiveness of the lignan-melatonin combination in blocking adrenal steroid hormone secretion at the cellular level and whether this combination would affect protein content of the steroidogenic enzyme aromatase in adrenal cells. Because many forms of human and canine disease have pathophysiologic similarities,[19] commercially available human adrenal carcinoma cells were chosen as a substitute for canine adrenal gland cells to evaluate adrenocortical function in the treatment conditions.

Materials and Methods

Cell culture—Human NCI-H295R adrenocortical carcinoma cells[a] were maintained in Dulbecco modified Eagle medium with nutrients,[b] 2.5% growth medium supplement,[c] and universal culture supplement[d] and cultivated at 37°C. The lignan phytoestrogens ENL[e] and END[e] were dissolved in dimethyl sulfoxide to a concentration of 10mM to create stock solutions. Aliquots of the stock solutions were frozen at –20°C. Melatonin[e] and cAMP[f] solutions at a concentration of 1mM were newly prepared for each experiment in sterile water. Cellular steroidogenesis was stimulated with cAMP.[20] Cells were seeded at a density of 3×10^6 cells/100-mm cell culture plate and separated into the following groups: untreated control, 100M cAMP (cAMP control), 100μM cAMP plus ENL plus melatonin (ENL treated), and 100μM cAMP plus END plus melatonin (END treated). Cells were treated for 24 and 48 hours. Cell culture medium was collected, and cells from each group were harvested at the 2 time points and frozen at –80°C until analyzed. This experiment was repeated 9 times.

Radioimmunoassay—Steroid hormones included in a canine adrenal profile test offered by the Clinical Endocrinology Service at the University of Tennessee were analyzed. Concentrations of progesterone,[g]

17-hydroxyprogesterone,[h] androstenedione,[h] aldosterone,[g] estradiol,[h] and cortisol[g] were determined in the cell culture medium by use of a radioimmunoassay method. Assays were performed in accordance with manufacturers' instructions.

Performance characteristics for each radioimmunoassay were determined for cell culture medium. Intra-assay and interassay coefficients of variation for the various radioimmunoassays were, respectively, as follows: progesterone, 4.1% and 7.0%; 17-hydroxyprogesterone, 6.7% and 17.2%; androstenedione, 5.5% and 7.9%; aldosterone, 5.5% and 6.6%; estradiol, 10.4% and 10.6%; and cortisol, 10.4% and 6.4%. Mean percentage recoveries of known amounts of hormones added to the medium were as follows: progesterone, 104.0%; 17-hydroxyprogesterone, 115.0%; androstenedione, 101.0%; aldosterone, 104.4%; estradiol, 97.0%; and cortisol, 102.0%. Serial dilution of cell culture medium yielded the following percentages of expected values: progesterone, 81.0%, 70.0%, and 71.0%; 17-hydroxyprogesterone, 111.0%, 99.0%, and 117.0%; androstenedione, 103.0%, 107.0%, and 125.0%; aldosterone, 108.0%, 113.0%, and 128.0%; estradiol, 106.0%, 113.0%, and 104.0%; and cortisol, 92.0%, 86.0%, and 83.0%.

Western immunoblot analysis—Cell pellets were incubated in lysis buffer[i] supplemented with protease inhibitors on ice for 10 minutes. Supernatants were isolated from cell lysates after centrifugation of crude lysates at $15,000 \times g$ for 20 minutes. After protein quantification, 20 μg of proteins from each lysate was resolved via electrophoresis in 10% SDS-polyacrylamide gels and transferred to a nitrocellulose filter. Nonspecific protein sites on the filter were blocked by incubation of the filter with 3% nonfat milk in NaCl-Tris-Tween 20 buffer (10mM Tris-HCl, pH 7.2; 150mM NaCl; and 0.05% Tween 20) at ambient temperature (approx 23°C) for 30 minutes, then the filter was incubated with an antibody specific for aromatase[j] (1:250) for 16 hours
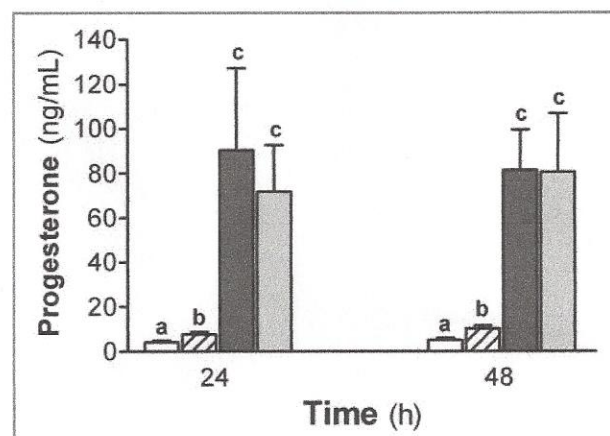

Figure 1—Mean ± SD progesterone concentrations in culture medium containing human adrenal carcinoma cells treated with medium alone (white bars), medium containing cAMP only (hatched bars), or medium containing cAMP and melatonin plus ENL (dark gray bars) or END (light gray bars) after 24 and 48 hours of cultivation. Values represent the results of 9 experiments/treatment. [a–c]For each time point, values with different letters are significantly (P < 0.05) different.

at 4°C. The filter was washed and incubated with a horseradish peroxidase–conjugated antibody[k] at ambient temperature for 45 minutes. The antigen-antibody complex on the filter was detected via chemiluminescence and visualized by means of autoradiography. Signals from the immunoblot were assessed by means of densitometry.

Statistical analysis—Differences in hormone concentrations based on treatment (untreated, cAMP control, ENL-treated, and END-treated cells) and time point (24 and 48 hours of cultivation) were evaluated by use of ANOVA with a randomized block design and least significant difference post hoc test. Statistical analysis was performed by use of commercially available software.[l] A value of $P < 0.05$ was considered significant.

Results

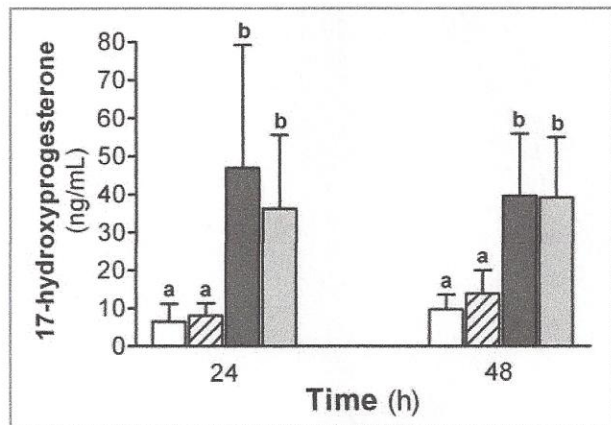Secretion of 4 of 6 steroid hormones was stimulated by the addition of cAMP to culture medium containing

Figure 2—Mean ± SD 17-hydroxyprogesterone concentrations in culture medium containing human adrenal carcinoma cells treated with medium alone (white bars), medium containing cAMP only (hatched bars), or medium containing cAMP and melatonin plus ENL (dark gray bars) or END (light gray bars) after 24 and 48 hours of cultivation. *See* Figure 1 for remainder of key.

Figure 3—Mean ± SD androstenedione concentrations in culture medium containing human adrenal carcinoma cells treated with medium alone (white bars), medium containing cAMP only (hatched bars), or medium containing cAMP and melatonin plus ENL (dark gray bars) or END (light gray bars) after 24 and 48 hours of cultivation. *See* Figure 1 for remainder of key.
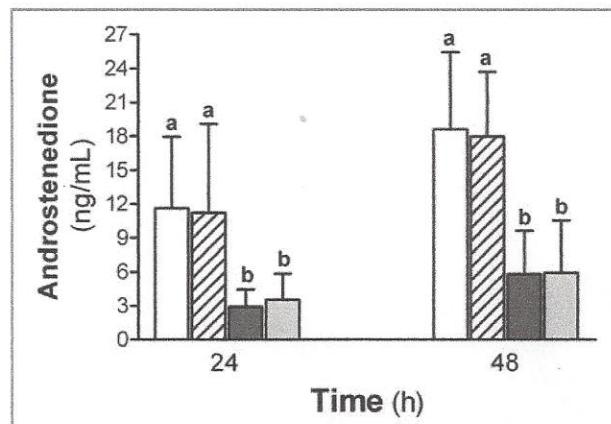
human adrenal carcinoma cells. Progesterone concentrations increased with addition of cAMP to the culture

Figure 4—Mean ± SD aldosterone concentrations in culture medium containing human adrenal carcinoma cells treated with medium alone (white bars), medium containing cAMP only (hatched bars), or medium containing cAMP and melatonin plus ENL (dark gray bars) or END (light gray bars) after 24 and 48 hours of cultivation. *See* Figure 1 for remainder of key.
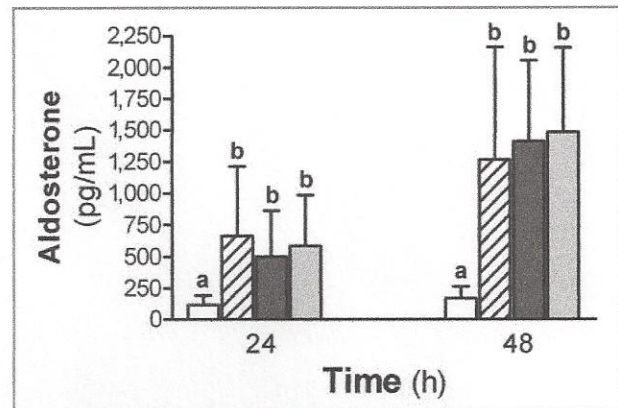
Figure 5—Mean ± SD estradiol concentrations in culture medium containing human adrenal carcinoma cells treated with medium alone (white bars), medium containing cAMP only (hatched bars), or medium containing cAMP and melatonin plus ENL (dark gray bars) or END (light gray bars) after 24 and 48 hours of cultivation. *See* Figure 1 for remainder of key.
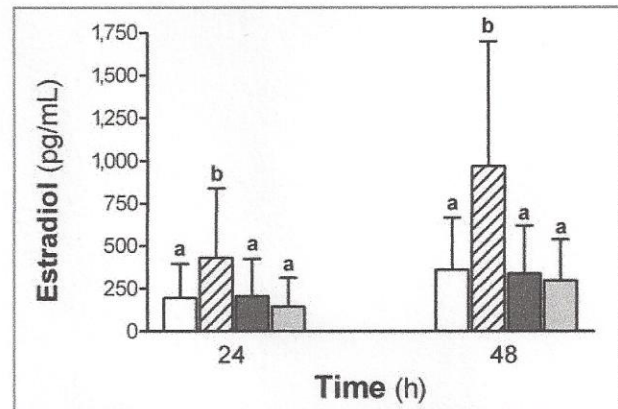
Figure 6—Mean ± SD cortisol concentrations in culture medium containing human adrenal carcinoma cells treated with medium alone (white bars), medium containing cAMP only (hatched bars), or medium containing cAMP and melatonin plus ENL (dark gray bars) or END (light gray bars) after 24 and 48 hours of cultivation. *See* Figure 1 for remainder of key.
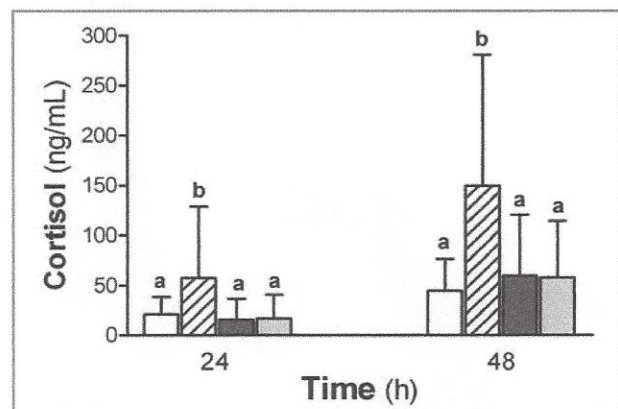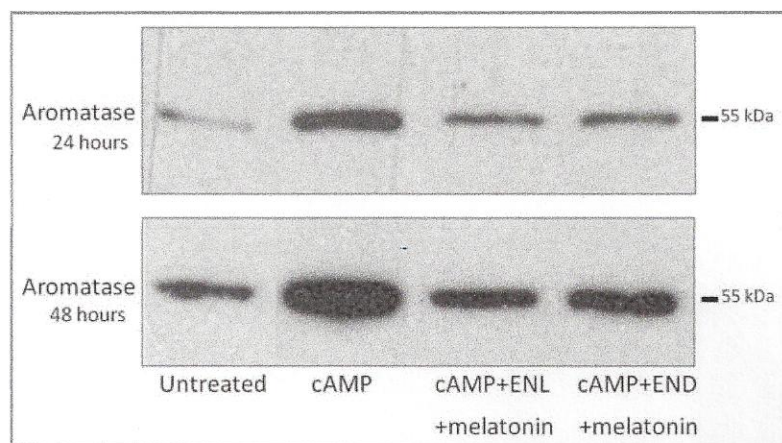
Figure 7—Representative immunoblots (3 separate experiments) displaying aromatase protein content in adrenal carcinoma cells treated with cAMP (100μM) and with a combination of lignan (100μM; ENL or END) and melatonin (100μM) for 24 and 48 hours. Aromatase content was analyzed by means of western immunoblotting with a specific antibody to detect the enzyme.

medium; however, there were no observable increases in 17-hydroxyprogesterone and androstenedione concentrations (Figures 1–3). In addition to progesterone, aldosterone, estradiol, and cortisol concentrations increased with cAMP stimulation (Figures 4–6).

The combination of ENL or END with melatonin in the culture medium of cAMP-stimulated cells resulted in significant increase in progesterone concentrations 24 and 48 hours after cultivation, compared with results for the cAMP-stimulated control cells. The cells responded similarly with increased 17-hydroxyprogesterone concentrations at 24 and 48 hours in cAMP-stimulated treatment groups, compared with concentrations in the cAMP-stimulated control cells. The combinations of ENL and END with melatonin in culture medium resulted in a significant decrease in androstenedione, estradiol, and cortisol concentrations in cAMP-stimulated cells; however, neither treatment combination had a significant effect on aldosterone concentrations, compared with the cAMP-stimulated control cells.

Addition of cAMP to the culture medium increased aromatase content by approximately 400%, compared with the content in untreated control cells (Figure 7). When lignan, melatonin, and cAMP were combined in the culture medium, there was a mean decrease in aromatase content to approximately 60% of the cAMP-stimulated value at 24 hours and to approximately 40% of the cAMP-stimulated value at 48 hours.

Discussion

Traditionally, cortisol is the only steroid hormone that has been measured in dogs suspected of having hyperadrenocorticism. However, dogs with hyperadrenocorticism, whether pituitary- or adrenal-dependent, may also have high amounts of adrenal sex hormones.[21] In dogs[22,23] and cats[2,24] with adrenal-dependent hyperadrenocorticism, blood concentrations of sex steroid hormones secreted by the adrenal glands are reportedly higher than reference limits; however, cortisol concentrations can be within or lower than reference limits.

In the present study, because of a lack of commercial availability of canine adrenal tumor cells, human H295R adrenocortical carcinoma cells were used to investigate the effect of lignans and melatonin on adrenocortical function. The H295R cells are capable of producing mineralocorticoids, corticosteroids, androgens, and estrogens and possess the enzymes involved in steroid hormone formation.[25,26] Basal cortisol concentration in the present study comprised 48.0% of steroid hormone production by H295R cells, followed by androstenedione at 26.6%, 17-hydroxyprogesterone at 14.7%, progesterone at 9.6%, estradiol at 0.4%, and aldosterone at 0.3%. The relative proportion of basal steroid hormone concentrations in the present study with H295R cells is similar to that found in initial blood samples from dogs with adrenal-dependent hyperadrenocorticism,[21] which suggests similarities in the steroid biosynthetic pathways of dogs and human H295R adrenocortical carcinoma cells. Furthermore, as in dogs and cats with adrenal tumors, humans with adrenal tumors can have high sex steroid hormone concentrations,[27,28] with cortisol concentrations within reference limits.[27] Given the similarities in adrenal steroid biosynthetic pathways and in adrenal tumor steroid hormone secretions, human H295R adrenocortical carcinoma cells appear to be an acceptable in vitro model for canine adrenal-dependent hyperadrenocorticism for the purpose of studying the effects of various treatments on adrenal steroidogenesis.

To the authors' knowledge, our study is the first to address the effects of a combination of lignan phytoestrogen and melatonin on cell secretion of multiple adrenal steroid hormones. Stimulation of cells with cAMP allowed evaluation of this combination on exaggerated hormone concentrations representative of the disease condition. Interestingly, 17-hydroxyprogesterone and androstenedione secretion did not increase with the addition of cAMP to the cell culture medium. The reason for this lack of stimulation is not known; however, it may have been attributable to increased conversion of 17-hydroxyprogesterone and androstenedione to cortisol and estradiol, respectively. Our results indicated the lignan-melatonin combination was effective in decreasing concentrations of androstenedione, estradiol, and cortisol in cAMP-stimulated adrenal tumor cells. Cell treatment with the combination decreased cAMP-stimulated estradiol and cortisol secretion to basal (control) values and androstenedione to less than basal values. In addition, in vitro treatment with the lignan-melatonin combination reduced the amount of aromatase protein, a pivotal enzyme in the conversion of androstenedione or testosterone to estradiol, in concert with a decrease in estradiol concentration.

Estradiol is one of the hormones commonly detected in high blood concentrations in dogs with atypical hyperadrenocorticism, and because it can be synthesized in various tissues,[4,5] treatment of affected dogs can be challenging. The suppression of aroma-

tase activity by lignan or melatonin is not specific to the adrenal gland because an inhibitory effect has also been detected in other cells and tissues such as MCF-7 breast cancer cells,[8] mammary tumors,[29,30] granulosa-luteal cells,[7] and preadipocytes.[31] Therefore, it is expected that the lignan-melatonin combination would be effective in decreasing estradiol secretion regardless of the tissue source. Interestingly, treatment with the lignan-melatonin combination increased progesterone and 17-hydroxyprogesterone concentrations in the cAMP-stimulated cells which, as with estradiol and androstenedione, are commonly high in dogs with atypical hyperadrenocorticism. This increase in progestin concentration has been documented by other researchers, who evaluated phytoestrogens in rat adrenocortical cell cultures[32] or melatonin in primate adrenal cell cultures.[13] The increase is reportedly due to a reduction in expression of cytochrome P450 c21-hydroxylase.[32] It is not known whether the combination of lignans ENL and END with melatonin inhibit the 21-hydroxylase enzyme in H295R cells; however, inhibition of this enzyme would be expected to increase progestins and decrease cortisol concentrations, as was observed in the present study. Inhibition of cytochrome P450 c21-hydroxylase would also be expected to cause a decrease in aldosterone concentration, which was not observed, and the reason for this is not clear.

Given in vitro results, it does not appear that the lignan-melatonin combination would be an appropriate treatment for dogs with atypical hyperadrenocorticism involving high blood progesterone or 17-hydroxyprogesterone concentrations. However, it is our experience that in dogs treated with a combination of lignan and melatonin, the combination does not cause an increase in progestins concentrations in all dogs and even decreases high concentrations in some. The response differences to lignan and melatonin among dogs is not unexpected given individual dog variability, whereas cell cultures provide a more homogeneous population that responds similarly over time without the influence of body tissue factors. It is also possible that the lignan-melatonin combination treatment affects steroid hormone secretion differently in dogs with pituitary-dependent hyperadrenocorticism versus dogs with adrenal-dependent hyperadrenocorticism; however, this possibility has not been evaluated clinically. Furthermore, it is not known whether treatment with the lignan-melatonin combination directly affects any steroidogenic enzymes or whether it may affect steroidogenesis through modulation of 1 or more signaling pathways, such as the extracellular signal-regulated kinase pathway. Others have reported that ACTH induces activation of Erk1/2 in human H295R adrenal cells[33] and that activation of the ERK cascade is involved in steroid hormone production by Y1 mouse adrenocortical cells.[34] In addition, several in vitro studies have shown that melatonin inhibits steroidogenesis by reducing the secreted or intracellular concentration of cAMP[35-37] and can affect both basal and stimulated cAMP production.[11,36]

We do not know whether the decrease in hormone concentrations in the present study can be attributed, in part, to melatonin inhibiting the stimulatory effect of the added cAMP. However, this possibility is unlikely because decreases were not detected in all hormones by the lignan-melatonin combination. The mechanism or mechanisms by which that combination modulates adrenal steroidogenesis remains to be clarified. The fact that treatment of human adrenal carcinoma cells with a lignan-melatonin combination resulted in a significant lowering of androstenedione, estradiol, and cortisol concentrations in cell cultures suggested that this combination may yield similar effects on adrenal steroid hormone secretion in dogs with atypical or typical hyperadrenocorticism.

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