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Urinary Excretion of Vanillylmandelic Acid in Rabbits

Jong Suh Kim* and Jae Baek Kim**

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家兎尿中 Vanillylmandelic Acid(VMA)排泄量의 變化에서 藥物反應과 circadian rhythm을 追求하기 爲하여 家兎에 對해 各種 stress를 加하고, 이에 依한 VNA値 增加에 對한 有效性을 몇가지 藥劑에서 찾기 爲해 電氣 stress를 미리 加한 家兎에 對하여 投與하고 이들 尿中 VMA値를 測定해 하였다.

正常狀態의 家兎 1日尿中의 VMA値는 平均 19.17×10^{-2}mg/24hrs였다. 電氣 stress를 加한 家兎尿中의 VMA値는 平均 28.87×10^{-2}mg/24hrs로 가장크게 增加하였고, 騷音 stress를 加하였더니 平均 27.39×10^{-2}mg/24hrs로 增加하였으며, 照明 stress를 加했을때는 平均 19.10×10^{-2}mg/24hrs로 거의 變化가 없었다.

電氣 stress를 加한 家兎에 reserpine을 投與했더니 平均 VMA値가 18.90×10^{-2}mg/24hrs로 排泄抑制效果가 가장 좋았고, 酸棗仁湯을 投與했을때 平均 25.76×10^{-2}mg/24hrs로 抑制되었으며, 人蔘엑기스를 投與했을때 平均 28.14×10^{-2}mg/24hrs로 效果가 거의 없었다.

It was the French physiologist Claude Bernard that defined[1] precisely the experimental methods to be applied on the living bodies in his great work "Introducton to Experimental Medicine". Hereby, has been introduced[2] a new concepts "Internal Environment" that is to be stabilized through adaptation-process to counter changes followed by stress-responses.

Thereafter, Oliver and Schafer[3] demonstrated the extracts of the adrenal glands had the powerful pressor effects, when these were injected into animals, and Walter Cannon, who expanded Bernard's theory showed[5-6] that epinephrine released from the adrenal glands was the main active substance responsible for such sympathomimetic actions, taking the central role involved in preserving the internal environment.

Also it was found by Cannon[4] the ability to preserve the internal environment vary under different general conditions and during the normal and pathologic ups and downs of existence in an ordinary life cycle, and the periodical rhythmicity of the adaptive

** College of Pharmacy, Won Kwang University
* Department of Pharmacy, Eul Ji Hospital

process has some relationship with the circardian secretory pattern of epinephrine.

Entering 20 century, many investigators[2] began to explain the adaptive ability to stress of the internal environment in the light of quantitative discharge of epinephrine.

Hans Selyed[7] called attention to endocrine system comprising hypothalamic-pituitary and adrenocortical relationships, controlling the adaptation process responding to stress. With such new concept, the most concern shift to the adrenal steroids and ACTH in the mechanisms underlying the stress-responses.

In the middle of the 20 century, scientisis definitely demonstrated that norepinephrine released from sympathetic nerve endings was a substance having a role as neurotransmitter in a certain part of the central nervous system, and then was followed dopamine to be defined as main neurotransmitter in the extrapyramidal system. With the availabilty of radioactive compounds and new techniques like spectrophotofluorometry, new interest was triggered in catecholamine research and more information was acquired concerning its biosynthesis, metabolism, disposition, excretion etc.,[8] indicating that they mediate secretion of the hypothalamic releasing factors that control pituitary function-it was inevitable that research on stress and catecholamines would again converge.

At the same time, it was proposed[9] that the conversion of tyrosine to catecholamine is catalyzed by the enzyme tyrosine-hydroxylase, which is believed to be the rate-limiting step in the biosynthesis of the catecholamines, and increase in the enzyme levels are mediated by cyclic AMP. As a result, neurcendocrinology has been merged with catecholamine and stress.

On the other hand, according to Kopin[2] Lennart and his associates have shown that there are many stresses in normal, every day life that increase catecholamine excretion in humans, and Halz[10], Ishihara[11], Birke[12] found a correlation between blood pressure and plasma catecholamine levels. Also, the results of Yoichi Serius' study[13] were interpreted to mean that catecholamines were secreted as a homeostatic so as to adjust the hemodynamic change and to keep the homeostasis. However, there has been indication that stress-induced changes in the CNS is different from the peripheral catecholamine responses to stress. Such differences are not yet clearly defined and these questions remain a challenge.

With such, it was found[14] that there exists a certain periodicity in the physiological phenomena of human body, and circardian rhythym of a regular fluctuation in catecholamine secretion with a period of approximate 24 hours. As a consequces, it was believed that there is circarian variation in drugabsorption, metabolism, excretion and drugtoxity (LD_{50}), therefore showing[15] the possibility of the choice of the correct dosage according to the circardian rhythm. Neverthelss, how far the circardian variation due to stress-responses influence on the the in-vivo drug action, and how far circardian variation in the effectiveness of drug may also be a result of diurnal rhythm in target susceptibility, are not well documented.

On the other hand, it has been understood how the neurotransmitter, catecholamines

such as norepinephrine, dopamine, control release and inhibition of the hypothalamic hormones and how a variety hormones are interrelated[16] therethrough.

The author's attention has been concentrated on the so far accmulated knowledge[17] that the biological periodicity exhibits a considerable variation under stress-conditions, and further, catecholamines must play one of the most important role[18] in controlling the biorhythm. In order to investigate any significant correlation between the interernal biological variation and urinary excretion of catecholamine, urinary catecolamines were measured in its metabolized form of VMA out of the urine collected from the animal under stress. And further such observation was made in the animals after some drugs having effets on catecholamine metabolism were administered.

EXPERIMENTAL

Materials and Reagents-Reserpine (U.S.P.), san-jo-in-tang, ginseng extract, vanillylmandelic acid (Sigma Co.), potassium carbonate, potassium ferricyanide, zinc sulfate, indole, phosphoric acid (85%), hydrochloric acid, sulfuric acid, ethanol (95%), ethyl acetate, toluene, VMA standard solution (VMA control solution, Sigma Stock No.480-1), potassium carbonate solution (0.4M), potassium ferricyanide solution (0.6w/v%), zinc sulfate solution (1.2w/v%) indole-phosphoric acid solution (Indole is dissolved in 95% ethanol to make 0.5% indole-ethanol. 1 ml of the indole-ethanol solution is diluted with 40 ml of 85% phosphoric acid of temperature 0-5°C, freshly prepared.), VMA woring standard solution (0.05mg/ml of VMA solution as prepared for working standard), hydrochloric acid (6N).

Animals -Male and female rabbits (1.6-2.1kg) were maintained individually in stainless steel cages for at least 2 weeks in the laboratory environment (12-hr dark cycle begining at 7 P.M. and controlled temperature and humidity). They were fed laboratory ration until 20-24 hours before an experiment.

Preparation of Extracts-50.6g of herbs (*parched jujube seed* 4: *Anemarrhena rhizoma* 3: *Cnidiixrhizoma* 1.5: *Glycyrrrhizae radic* 2: *pachymaefungus* 3) were extractted with 1,000ml of distilled watrer on water bath and the filtrate was concentrated to about 120ml (son-jo-in-tang). 37.5g of 6-year Korea ginseng radix (Kum san origin) graded "30 pieces", was extracted with 1,000ml of distilled water on water bath and the filitrate was concentrated to about 120ml. (Ginseng extract).

Experimental Method-*Electric stress*·Manual type of telephone set was employed to produce electric stress of 24 voltage. Such electric stress was given to rabbits in the ear for 5 seconds each time with the interval of 1 hour, 6 times a day.

Noise stress. Average 80 dB noise was produced from a radio to stress the animal for 1 minute per hour, 6 times a day.

Light stress. Electric bulb of 100 voltage & 60 watt was employed to hinder sleep through continuous illumination with distance of 1m for 12 hours during a night.

Medication. The groups which electric stress was given to, were administeried by the following medication.

San-jo-in-tang. Each 4ml of the drug was dosed orally 3 times a day.

Ginseng extract. Each 4ml of the drug was dosed orally 3 times a day.

Reserpine. Each animal was injected intravenously in the ear with 0.1mg per Kg.

VMA Measurement. Experimental procedure by Sunderman was employed to measure VMA value in urine of the rabbits.

RESULTS AND DISCUSSION

Urinary VMA Value-In the laboratory environment rabbits showed 19.17×10^{-2}mg/24hrs and under electric stress 28.87×10^{-2}mg/24 hrs, about 50% increase above normal figure. In case of noise stress 27.39×10^{-2}mg/24hrs was shown, about 43% increase. And in case of electric illumination was given with 100 voltage and 60 watt to hinder sleep, the value turned out as 19.10×10^{-2}mg/24hrsas almost much as normal value.

As shown in table I where electricity, noise and electric light were used as stress, the electric stress demonstrated the highest VMA value. And the groups to which electric stress was given were selected to observe any change in VMA value shown after medication; as drug, were employed *san-jo-in-tang,* ginseng extract and reserpine, each of which was dosed to each animal. The changes shown are as in table Ⅱ where the VMA value is 18.90×10^{-2}mg/24hrs in reserpine group, indicating a decrease below the normal value(19.17×10^{-2}mg/24hrs); 25.76×10^{-2}mg/24hrs in *san-jo-in-tang* group, showing a slight decrease and 28.14×10^{-3}mg/24hrs in ginseng extract group, showing almost the same level to the case of electric stress. The statistical methods were employed to confirm the above findings by means of P-value. Table Ⅱ indicates that reserpine group is statistically most significant with $P<0.001$, *san-jo-in-tang* group significant with $P<0.025$ and ginseng extract group nonsignificant. Another experiment demonstrated that it took average 5 days for the rabbits given electric stress to begin to show the normal urinary VMA value once they had.

Figure 2 shows the comparison in the changes of each urinary VMA value between the case of electric stress only, and that of medication further such as *san-jo-in-tang,* ginseng extract and reserpine after electric stress.

Circardian rhythms have been described in numerous biological variables. Some of the better known examples are catecholamine secrection and steroidal hormone sercetion physiologically adjusted in correlation with the internal environment including endocrine-, brain-, and circulatory-functions. Consequently, a circardian rhythm has been discovered to take a positive role in controlling the fluctuation of daily disease state. Especially, catecholamine and serotonin have been respectively studied[14] in correlation with mental illness. Also, the concept homeostasis that the internal environment is

Table I—Urinary Excretion of VMA in Rabbits

Rabbit No.	Urine Volume ml/24hrs	Treatment, Stress(10^{-2}mg/24hrs) Control	Electrical	Noise	Light
1	65	21.6	32.3	28.3	21.3
2	60	17.6	28.4	28.0	17.7
3	72	15.7	27.1	26.2	15.4
4	18	20.3	31.2	29.3	20.1
5	64	18.7	24.9	26.1	17.7
6	86	19.3	27.6	25.0	18.9
7	75	19.1	27.2	28.4	1..5
8	86	18.6	28.0	25.5	18.8
9	56	20.1	31.2	29.2	21.1
10	66	21.2	32.0	31.0	22.2
11	74	17.3	30.5	27.5	17.5
12	70	20.2	28.3	26.9	17.3
13	81	18.1	25.7	26.1	18.5
14	62	17.3	26.4	25.1	17.0
15	79	21.1	31.8	30.1	20.3
16	84	21.4	29.4	27.1	21.8
17	64	19.2	27.8	26.4	19.5
18	71	18.3	29.8	26.9	19.2
Mean	72	19.17	28.87	27.39	19.10

Table II—Urinary Excretion of VMA in Electric Stress Rabbits Dosed Orally with San-jo-in-tang, Panax Ginseng Ext. and Reserpine I. V. injection

Rabbit No.	Treatment (10^{-2}mg/24hrs) Control (elcctricaJly stressed) San-jo-in-tang	Ginseng Ext.	Reserpine	San-jo-in -tang[a)]	Ginseng Ext. [a)]	Reserpine I. V. [b)]
1	27.2	25.7	32.3	24.1	27.1	21.5
2	28.0	26.4	28.4	25.2	26.0	19.4
3	31.2	31.8	27.1	26.7	30.1	16.0
4	32.0	29.4	31.2	27.2	29.2	20.1
5	30.5	27.8	24.9	25.1	27.9	18.9
6	28.3	19.8	27.6	26.3	28.5	17.5
Mean	29.54	28.48	28.58	25.76	28.14	18.90
S.D.		2.37		1.23	1.69	2.08
				259.89[c)]	79.67[d)]	896.78[c)]

a) 4ml, three times a day, *b*) 0.1mg/kg, *c*) Significantly different from control values at P<0.00 I, *d*) Nonsignificant at p<0.001 (compared to control)

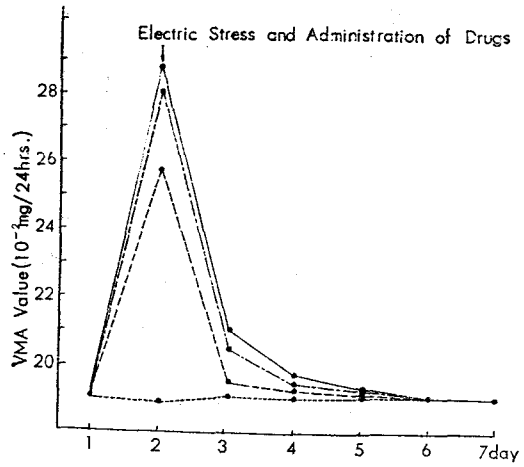

Figure 1-Normalization of the elevated urinary VMA value in rabbits.

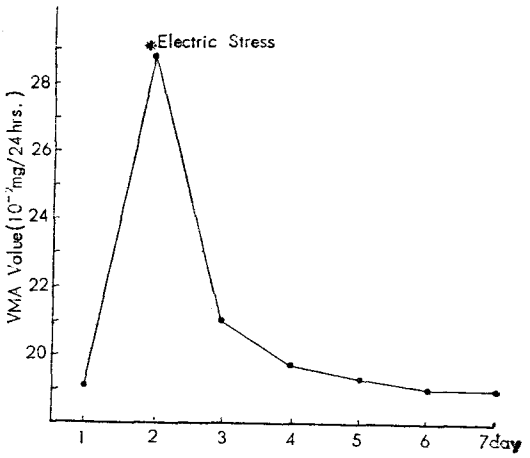

Figure 2-Urinary exer etion of VMA after electric stress and administration of durgs in rabbite. Key: ——, electric stress; ·····, reserpine injection; – – –, san-jo-in-tang; ——, pa-nax ginseng ext.

maintained in the steady state independent of all external variation, was introduced.

Many scientists demonstrated that when an animal is frightened or given pain, epinephrine is released[2-6] to withstand challenge. Epinephrine discharge takes an indirect pathway through by hypothalamus to take a positive role on the ACTH secretion at anterior pituitary. Such stimulating hormone is stimulating the adrenocortical hormonal action and exerting stimulative action on the steroids controlling carbohydrate metabolism. Adrenocortical hormone stimulates glycogen-production in the liver, which are under stress-state supplied and utilized through a variety of hormonal function to maintain homeostasis.

On the other hand, it was ascertained by Armstrong[19] that abnormal increase of urinary catecholamine excretion indicates a pheochromocytoma and neuroblastoma in human. In case of ordinary people, urinary VMA was 0.7~6.8mg/24hrs, while the figure was abruptly increased to 11~15mg/24hrs in case of pheochromocytoma and 22~58mg/24hrs[20-21] for neuroblastoma.

In the present study, the urinary VMA of normal rabbit was measured to be 0.157 ~0.216mg/24hrs, which may turn out 6.7mg/24hrs, when extrapolated to 60kg weigh of human, showing the similarity to the VMA figure of normal state of the latter. When electric stress was given to rabbit having the normal value of VMA, the urinary VMA was increased to minimum 0.249mg/24hrs and maximum 0.323mg/24hrs, expressing as 50% increase above the mean level during normal state. Such figure is corresponding to the mean value of 9.13mg/24hrs when extrapolated to 60kg of man,

which means lower level but considerably more increased than that of urinary VMF in case of pheochromocytoma.

When the animal was exposed to 80 dB noise, which would cause a temporary hearing loss to man, VMA was shown to be 0.250~0.310mg/24hrs. This indicates a considerable increase in tension due to stress, lower as it is than that due to electric stress.

When rabbits were hindered to sleep, the urinary VMA was 0.154~0.222mg/24hrs, which is not more than under normal state. Author interprets such phenomena in that rabbits had a good adaptability to light stress.

The group having received electric stress which caused the highest increase in nrinary VMA excretion, was selected as control group, and to a subject group of rabbit given the same stress, reserpine was intravenously injected in ear. As a result, the latter showed a lowered VMA value of 0.160~0.215mg/24hrs significantly P-value ($P<0.001$).

And also to the group given electric stress was a herbal tranquilizer called "san-jo-in-tang[22]" dosed, VMA excretion was lowered to 0.241~0.272mg/24hrs with significantly ($P<0.025$). But in case of the group to which ginseng extract was dosed, urinary VMA was 0.260~0.301mg/24hrs nonsignificantly.

CONCLUSIONS

In order to investigate drug circardian rhythm on a rational basis, at first, urinary excretion of VMA were studied in rabbits given various stresses (electricity, noise and light), which were observed to have an increase in VMA value. Then to confirm the statistical significance of such increase, urinary VMA value were measured in rabbits given electric stress and then administered with various drug-medication (*san-jo-in-tang*, ginseng extract, and reserpine). The findings are as like the followings;

1. The daily urinary VMA excretion was measured be average 19.17×10^{-2}mg/24hrs in rabbits under normal state.

2. The VMA value was about 28.87×10^{-2}mg/24hrs in case of rabbits given electric stress, an increase to approximately 27.39×10^{-2}mg/24hrs in case of animals given noise-stress, and no change in case of light-stress with approximately 19.10×10^{-2}mg/24hrs.

3. The group which was given electric stress and then reserpine, showed VMA excretion value to be approximately 18.90×10^{-2}mg/24hrs, indicating the highest inhibitory effect on urinary VMA excretion, the other group to which san-jo-in-tang was dosed, showed the lowered mean value of 25.76×10^{-2}mg/24hrs, and from the group given gineseng extract was measured the mean VMA value to be 28.14×10^{-2}mg/24hrs, showing almost no inhibitory effect.

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