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Full Length Research Paper

Exogenous cycilc AMP and cycilc GMP influence the metabolism of traces of tritium-labeled glycerol in rabbits

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To characterize the dynamic distribution and metabolism of endogenous glycerol *in vivo*, the exogenous glycerol labeled by radioactivity was mimicked to trace its kinetics in Chinese Haerbin white rabbits. Based on the kinetic alternations, the metabolic equation of ^{3}H-glycerol was formulated. The determining coefficient (R^2) was 0.9876 closed to predicted value ($p < 0.05$). The proposed equation fits well with the metabolic dynamics of exogenous ^{3}H-glycerol within 30 h after injection. Moreover, according to the equation, the exogenous ^{3}H-glycerol accumulates mainly in kidney, intestine and fat after 95 h injection. Also the dynamic concentration of ^{3}H-glycerol in the examined kidney to intestine to fat and other tissues decreases gradually, indicating a underlying kinetic feature in Chinese Haerbin white rabbits. In addition, the applied exogenous cAMP and cGMP show a distinct impact on glycerol distribution.

Key words: ^{3}H-Glycerol, Haerbin white rabbits, trace kinetics, cAMP, cGMP.

INTRODUCTION

Cyclic nucleotides (CNT) are non-specific substances of low molecular weight that exist widely in organism and exhibit very important function in the course of vital movement and metabolism. Cyclic adenosine monophosphate (3´,5´-cycilc AMP, cAMP) and cyclic guanosine monophosphate (3´,5´-cycilc GMP, cGMP), as CNT belongings, are both called second messenger. And since 1957 and 1963, cAMP and cGMP have been discovered and purified, respectively. CNT have been reported to have important functions in regulating and controlling the cell growth and reproduction (Simpson et al., 2007; Taminato et al., 2002; Chen et al., 2005; Kang et al., 2005). It is related to the various physiology, bio-chemistry and metabolism in animal (Han et al., 2004; Saeki and You, 2003; Holz et al., 2008; Ho and Chik, 1995; Sheriff et al., 1997; Julie and Robert, 2008). The study *in vivo* showed that the radioactive labeled substances had the same metabolic disciplinarian as endogenous substances which had the same molecular structure (Chase and Rabinowitz, 1967; Joseph and Robert, 1966). It could reveal the dynamic features of physiology, biochemistry and pharmacology by observing the dynamic disciplenarian of its absorption, distribution, transformation, metabolism and final egestion. Amino acids, typical of leucine, labeled with stable isotopes have been used as endogenous tracers for the study of protein and lipid metabolism since mid 1980s (Cryer et al., 1985; Schaefer et al., 1992). (Joseph and Robert, 1966) found the importance for metabolism by rat adipose tissue using by ^{14}C and ^{3}H labeled glucose. Different stable isotope-labeled compounds (e.g., [^{15}N]glycine, [^{13}C] phenylalanine, [^{13}C] leucine and [^{3}H] leucine) have been used in tracer studies. The results suggested that all of these act in a similar manner as a tracer (Lichtenstein et al., 1990).

At present, there is lack of research done on the metabolism of glycerol using isotope. To investigate the biotransformation process of glycerol in animal tissues, we applied isotopic tracer technique which was aided with compartmental models to study the distribution and metabolism of ^{3}H-glycerol in Haerbin White rabbits. The result will provide the basis on the controlling of lipid metabolism to be implemented on larger animal species, such

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as cattle, sheep and goat.

MATERIALS AND METHODS

Experimental animals and chemicals

A total of 24 Haerbin white rabbits (male, body weight of 1500 ± 200 g) at age of 2 - 3 months old were from the experimental animal center of the Fourth Military Medical University (Xi'an City, Shaanxi Province, China). The rabbits had unrestricted access to the food and water. In addition to the compound feed according to feeding standard each day, carrots and cabbages were added each time after bleeding. All animal procedures were performed according to protocols approved by biological studies animal care and use committee P. R. China. Meanwhile, cAMP and cGMP were both supplied by Sigma Co., USA. ^{3}H-Glycerol was supplied by GE Healthcare Bioscience (the specific radioactivity was 18.5 - 37 GBq/mmol, the activity concentration was 37 MBq/mL, the intensity was 37 MBq. Its storage condition was -20℃ in liquid.).

Experimental treatments

24 experimental rabbits were randomly divided into 4 groups. All animals were injected with 1 ml of 1.48 MBq/ml ^{3}H-glycerol intraperitoneal injection resolution. In addition, group χ rabbits, the control group, were hypodermically injected with 1 ml of 9 mg/l saline infusion solution. Group χ rabbits were hypodermically injectted with 1 ml of 0.64 mg/ml cAMP intraperitoneal injection solution. Group χ rabbits were hypodermically injected with 1 ml of 0.64 mg/ml cGMP intraperitoneal injection solution. Group χ rabbits were hypodermically injected with 1 ml of 0.32 mg + 0.32 mg/ml cGMP+cAMP intraperitoneal injection solution.

2 ml of blood were taken from ear-side vein from 3 rabbits in each group at 1, 3, 6, 10, 15, 21, 28, 36, 45, 55, 65, 75, 85 and 95 hours at post injection. The blood was collected into EDTA treated centrifuge tubes. After centrifugation, the plasma was stored at -78℃. The tissue samples of heart, liver, spleen, lung, kidney, brain, stomach, intestine, muscle (from thighs), fat (around viscera), bladder and didymus, were taken from the remaining 3 rabbits in each group. All tissue samples were stored at -78℃ immediately after collection.

^{3}H-Glycerol measurement in blood

For each 1 ml of plasma sample, 300 µl of 60% $HClO_4$, 600 µl of 30% H_2O_2 and one drop of isopropanol were added. The mixture was digested at 70℃ -80℃ for 3 - 4 h. The volume of 900 µl digested sample were transferred into a scintillation cup and mixed with 10 ml of scintillating solution and shook until the mixture was clarified. The samples were incubated in the dark for 8 - 10 h to reduce errors. The liquid scintillation counter was used to measure the concentration of ^{3}H in the samples.

^{3}H-Glycerol measurement in tissues

For each 10 mg of smashed tissue samples, 300 µl of 60% $HClO_4$, 600 µl of 30% H_2O_2 and one drop of isopropanol were added. The mixture was digested at 70℃ -80℃ for 3 - 4 h, the volume of 900 µl digested sample were transferred into a scintillation cup. The digested sample was mixed with 10 ml of scintillating solution and shook until the mixture is clarified. The samples were incubated in the dark for 8-10 h to reduce errors. The liquid scintillation counter was used to measure the concentration of ^{3}H in the samples.

Establish tracing dynamics model

According to theory and methods of tracing dynamics, we established ^{3}H-glycerol tracing dynamics model utilizing the bicameral model and compared the theoretical value and actual value of blood concentration based on this model, insured the fitting degree of accuracy and reliability of the model using F-test.

We analyzed the control group with the Marquardt method and used the traditional metabolism bicameral model $\hat{Y}_{(t)}=Ae^{-\alpha t}+Be^{-\beta t}-(A+B)e^{-kt}$ to modify the model as $\hat{Y}_{(t)}=Ae^{-\alpha t}+Be^{-\beta t}-Ae^{-\gamma t}-Be^{-\delta t}+C$. In the 2 models: $\hat{Y}_{(t)}$ is the predictive value (Bq/ml), A is the central compartment's initial concentration (Bq/ml), B is the peripheral compartment's initial concentration (Bq/ml), C is the concentration constant (Bq/ml), k is the absorption rate constant, α is the distribution rate constant, β is the remove rate constant, γ is the central compartment's absorption rate constant, δ is the peripheral compartment's absorption rate constant, and t is the time (h).

Statistical analysis

Compartmental models and dynamic parameters were established by nonlinear regression analysis and marquardt analysis (Barrett et al., 1998; Cobelli and Foster, 1998). Paired data means were compared by anova and subsequent Duncan's multiplerange test and samples t-test was used for the comparison within the same treatment. Statements of statistical significance were based on $p < 0.05$ and $p < 0.01$, using the statistical sofware SPSS version 12.0.

RESULTS

^{3}H-Glycerol in blood samples

Table 1 summarized the ^{3}H-Glycerol changes in blood after intraperitoneal injection treatment. In comprison to the control group, the cGMP group did not show significant differences at each time points except the time points between 10 h and 15 h ($p < 0.05$). The cAMP group showed significant difference ($P < 0.05$) and extremely significant difference ($p < 0.01$) from 21 to 75 h, the cAMP group showed stronger radioactivity strength than the control group. Compared with the control group, the cGMP + cAMP group showed significant difference ($p < 0.05$) and extremely significant difference ($p < 0.01$) at all time points except 55 h at post injection. Moreover, this group had comparatively weaker radioactivity strength than the control group.

^{3}H-Glycerol blood dynamic model to central

Through computer iterative analysis, F-test indicated that fitting result could fit the modified bicameral model successfully, and the best mathematical equation to reflect the model was: $\hat{Y}_{(t)}=47{,}500.10081e^{-0.101002694t}+178.2001885e^{-0.072195884t}-47{,}500.10081e^{-0.100728077t}-178.2001885e^{-0.170750988t}+8.923484752$. The coefficient of determination $R^2 = 0.9876$. In Figure 1, the predictive value is closed to the measured value ($p > 0.05$) and the curve fitting result of ^{3}H-glycerol was relatively ideal. Furthermore, it showed

Table 1. Results (mean ± SD) of radioactivity mensuration of ^{3}H-glycerol blood sample.

Post injection time (h)	Control group (Bq/ml)	cGMP group (Bq/ml)	cAMP group (Bq/ml)	cGMP+cAMP group (Bq/ml)
1	12.98 ± 1.33	9.82 ± 0.63	13.82 ± 1.08	5.95 ± 0.56**
3	16.50 ± 1.35	13.02 ± 1.26	16.67 ± 1.28	10.92 ± 0.81**
6	18.12 ± 1.60	16.50 ± 1.60	19.80 ± 1.38	8.45 ± 0.93**
10	15.42 ± 1.12	13.02 ± 1.36*	17.15 ± 1.63	8.25 ± 0.59**
15	12.52 ± 0.76	7.43 ± 0.34*	13.92 ± 1.53	7.10 ± 1.09**
21	10.02 ± 1.39	10.88 ± 0.96	18.08 ± 1.47**	6.85 ± 0.79*
28	9.20 ± 0.66	8.52± 0.78	13.28 ± 1.32*	5.13 ± 0.38*
36	10.02 ± 0.70	9.92 ± 0.73	24.72 ± 1.48**	5.72 ± 0.51**
45	9.15 ± 0.48	11.80 ± 0.79	13.28 ± 1.54*	5.47 ± 0.57*
55	9.00 ± 0.69	9.73 ± 0.78	15.68 ± 1.57**	7.78 ± 0.76
65	9.42 ± 0.40	10.3 ± 0.27	12.72 ± 1.36*	5.67 ± 0.48*
75	9.65 ± 1.39	9.12 ± 0.58	12.05 ± 1.20*	4.77 ± 0.27**
85	9.47 ± 1.09	7.83 ± 0.54	11.02 ± 1.14	4.58 ± 0.30**
95	8.58 ± 0.36	7.88 ± 0.29	9.80 ± 1.07	4.02 ± 0.37**

P is the level of significance. * *P < 0.05* as compared with control group in the same row; ** *P < 0.01* as compared with control group in the same row.

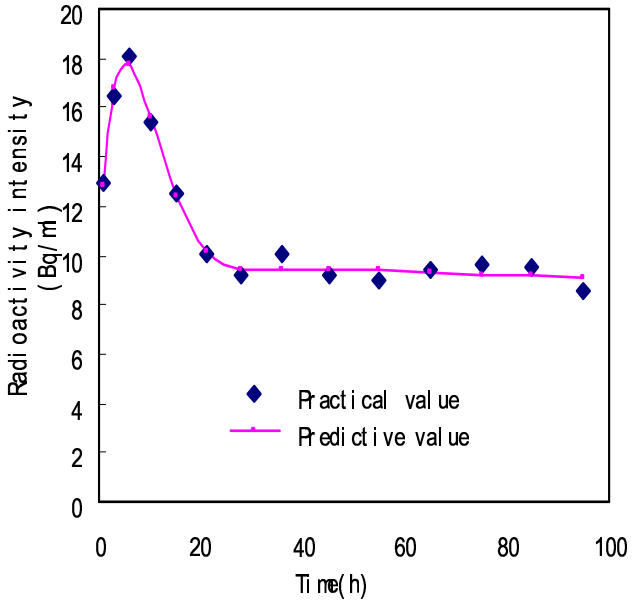

Figure 1. Dynamical graph of ^{3}H-glycerol in control group.

the model can be better mathematical equation to describe the metabolism ^{3}H-glycerol within 30 h after injection.

Figure 1 and Table 2 summarized the correlation among the 7 kinetic parameters. Judging by the Figure 1 and Table 2, the distribution rate constant (α) of ^{3}H-glycerol *in vivo* was 0.1010 h^{-1}, the distribution half-life ($T_{1/2}\alpha$) was 6.8627 h; the remove rate constant (β) was 0.0722 h^{-1}, remove half-life ($T_{1/2}\beta$) was 9.6009 h; the central compartment's absorption rate constant (γ) was 0.1007 h^{-1}, the absorption half-life ($T_{1/2}\gamma$) was 6.8814 h; the peripheral compartment's absorption rate constant (δ) was 0.1708 h^{-1}, the absorption half-life ($T_{1/2}\delta$) was 4.0594 h and it reached its peak at (T_{max}) 5.4704 h at the concentration of (Y_{max}) 17.8602 Bq/ml. The area under the curve (AUC) was 988.5864 Bq·h/ml. Furthermore, the correlation degree of the central compartment initial concentration, distribution constant and absorption rate constant closed to the complete correlation. Equivalently, the correlation degree of the peripheral compartment initial concentration, distribution constant and absorption rate constant closed to the complete correlation.

Figure 2 showed that the equation of cGMP was: $\hat{Y}_{(t)} = 3{,}487{,}998.211e^{-0.100008346\ t} + 1{,}196.1380e^{-0.074257678t} - 3{,}487{,}998.211e^{-0.09998773t} - 1{,}196.1380e^{-0.138834905t} - 6.8899465$, the coefficient of determination (R^2) was 0.7173.

Figure 3 showed that the equation of cAMP was: $\hat{Y}_{(t)} = 1{,}151{,}334.704e^{-0.099999727t} + 82.7320e^{-0.043981697t} - 1{,}151{,}334.704e^{-0.0100000349t} - 82.7320e^{-0.050899182t} - 11.66321682$, and the coefficient of determination (R^2) is 0.3064.

Figure 4 showed that the equation of cGMP + cAMP was: $\hat{Y}_{(t)} = 1{,}533{,}260.807e^{-0.099999942t} + 19.2961e^{-0.032746638t} - 1{,}533{,}260.807e^{-0.099998375t} - 19.2961e^{-0.429715906t} - 2.9276821$, and the coefficient of determination (R^2) is 0.7511.

The predictive values had great differences from measured values ($p < 0.01$) (Figures 2, 3 and 4). The addition of CNT leads to significant changes to the metabolism of glycerol ($p < 0.05$) and the effect of cAMP was the greatest of them.

^{3}H-Glycerol tissues dynamic model

The radioactive measurements of ^{3}H-glycerol in 12

Table 2. Correlation of dynamical parameter of ^{3}H-glycerol.

Parameter	A	α	B	β	γ	δ	C
A	1.0000	-0.9983	0.5034	0.4322	0.9985	-0.5554	-0.1682
α	-0.9983	1.0000	-0.4527	-0.3787	-0.9936	0.5070	0.1165
B	0.5034	-0.4527	1.0000	0.9910	0.5492	-0.9971	-0.7985
β	0.4322	-0.3787	0.9910	1.0000	-0.9793	-0.9793	-0.8584
γ	0.9985	-0.9936	0.5492	0.4809	1.0000	-0.5989	-0.2162
δ	-0.5554	0.5070	-0.9971	-0.9793	-0.5989	1.0000	0.7686
C	-0.1682	0.1165	-0.7985	-0.8584	-0.2162	0.7686	1.0000

A is the central compartment's initial concentration; *α* is the distribution rate constant; *B* is the peripheral compartment's initial concentration; *β* is the remove rate constant; *γ* is the central compartment's absorption rate constant; *δ* is the peripheral compartment's absorption rate constant; and *C* is the concentration constant.

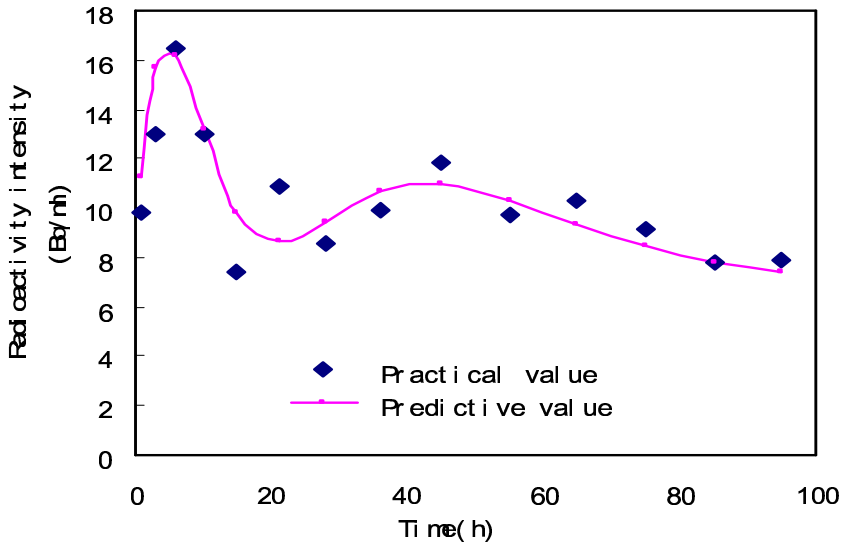

Figure 2. Dynamical graph of ^{3}H-glycerol in cGMP group.

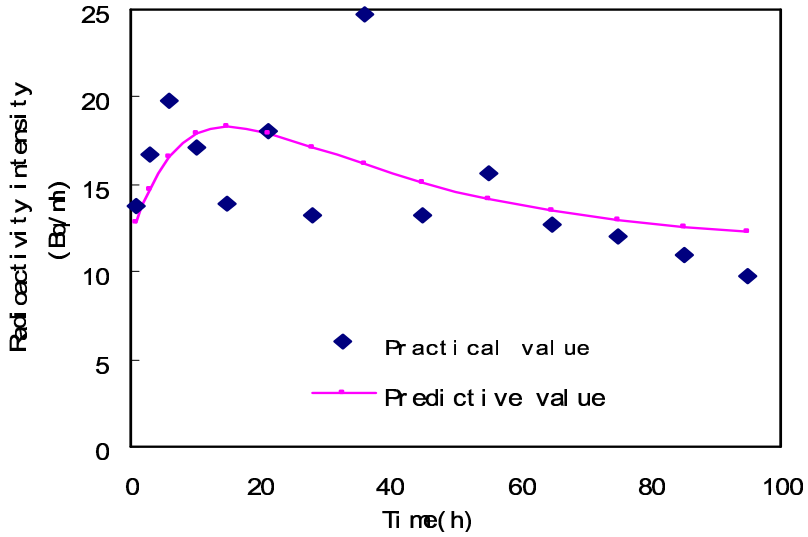

Figure 3. Dynamical graph of ^{3}H-glycerol in cAMP group.

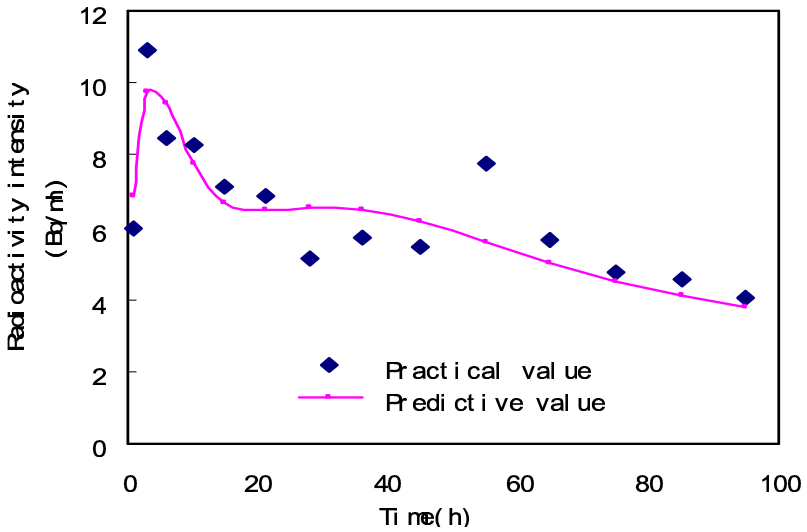

Figure 4. Dynamical graph of ^{3}H-glycerol in cGMP+cAMP group.

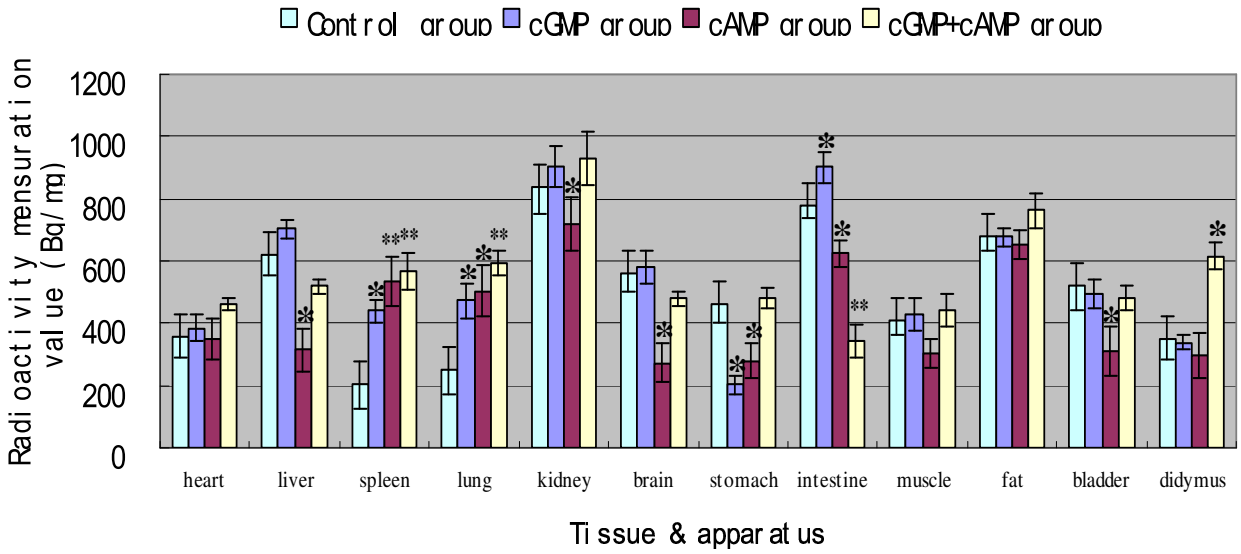

Figure 5. Results (mean ± SD) of radioactivity mensuration value of ^{3}H-glycerol in tissue samples. * $P < 0.05$ means as compared with control group in the same tissue & apparatus. ** $P < 0.01$ means as compared with control group in the same tissue and apparatus.

organs and tissues at the 95 h post-injection were showed on Figure 5. The part of the exogenous glycerol discharged by metabolism in course of transferring and a majority of glycerol was involved in composing of triglyceride, cholesterol and acetone body and was deposited in kidney, intestines, fat, liver, brain and many other tissues or organs. In control group the abundance of ^{3}H-glycerol was distributed in the order of kidney, intestine, fat, liver, brain, bladder, stomach, muscle, heart, genitals, lung, spleen with kidney had highest level of ^{3}H-glycerol. Meanwhile, the cyclic nucleotide had significantly changed ($p < 0.05$) and extremely significantly changed ($p < 0.01$) in the tissues of liver, lung, spleen, kidney, brain, stomach, intestine after hypodermic injection.

In cGMP group the distribution trend orders of ^{3}H-glycerol were kidney, intestine, liver, fat, brain, bladder, lung, spleen, muscle, heart, genitals and stomach. In the sequence, the positions of kidney, intestines, brain and bladder were not altered, but the orders of lung, spleen and stomach were altered distinctly ($p < 0.05$).

In cAMP group the distribution trend orders of ^{3}H-glycerol were kidney, fat, intestines, spleen, lung, heart, liver, bladder, muscle, genitals, stomach and brain. In the sequence, the positions of kidney and didymus were not altered, but the orders of spleen, lung, liver, stomach and brain were altered distinctly ($p < 0.05$).

In cGMP+cAMP group the distribution trend orders of ^{3}H-glycerol were kidney, fat, genitals, lung, spleen, liver, stomach, bladder, brain, heart, muscle and intestines. In the sequence, the positions of kidney and stomach were not altered, but the orders of didymus, lung, spleen, brain, muscle and intestines were altered distinctly ($p < 0.05$).

DISCUSSION

The commonly used radioactive isotopes are ^{3}H, ^{14}C, ^{15}N, ^{32}P, ^{35}S, ^{125}I, and ^{31}I (Chase and Rabinowitz, 1967). The half-life of ^{3}H is 12.33 years and do not need to regulate when it is applied in agrobiological experiment. It can only release β-ray that is quite easy to measure with advantage of high sensitivity, reliability, stability and safety. It is also a essential element which is contained in both organic substance and mineral (Hamawy, 1995). National radioactive sanitary standards are equivalent annual consumption < 185 GBq (Board on Radiation Effects Research, 2001; Bohner et al., 1983). Based on the previous studies and a great deal of experimental tests, we injected rabbit with1.48 MBq/ml per animal. It did not cause any radiation damage to the body that indicated that the test was carried out in a natural physiological status.

The radioactivity of ^{3}H-glycerol measured in the blood samples indicated that the cAMP group has a great effect on the metabolism of glycerol in the samples collected between 21 and 75 h at post injection and the cGMP group does not affect any changes. Since the significant decrease of ^{3}H-glycerol was observed in cAMP + cGMP group. It indicated that cGMP has negative effects on cAMP. cAMP can cause changes of many types of hormones *in vivo*. In this way, it helps to decompose triglyceride into glycerine and fatty acid, thus reduce the triglyceride and other fat in organs which need lots of blood supply. External ^{3}H-glycerol will not synthesize fat such as triglyceride. As a result, the concentration of ^{3}H-glycerol increases. The function of cGMP differs from that of the cAMP and has little effects on the glycerol metabolism. If the 2 are mixed together, the effects will be greatly improved. Glycerine only decomposes into 1-glycerol phosphate when there exist glycerokinase and energy supply. Then it is decomposed into phosphodihy droxyacetone and 3-glyceraldehyde phosphate. At last, the glycolysis begins and pyruvic acid is generated. Acetic coenzyme A is also generated. Tricarboxylic acid cycle and reversed glycolysis can cause gluconeogenesis, thus produce glucose hepatin.

The distributions in sample tissues are different between different groups. Spleen, lung and intestine are most vulnerable tissue types affected by cAMP, cGMP and cAMP + cGMP, with increased fat degradation in most cases. There is no glycerokinase in fat tissue. As a result, fat tissue cannot use glycerin. Glycerin generated by decomposing fat needs to go through the liver for further decomposition. Generally, medicine concentration reduces in the brain. This is owing to blood-brain barrier. However, some fat-dissolvent medicine can circumvent the blood-brain barrier and enter cerebrospinal fluid. In this way, the amount of the medicine in the brain increases.

The above reasons can cause external ^{3}H-glycerol to transform regularly in the blood. However, such regularity can be upset by injecting CNT. Injecting cGMP alone will greatly affect the transforming process ($p < 0.05$) and increases ^{3}H-glycerol. However, if cAMP and cGMP are injec-ted at the proportion of 1:1, the ^{3}H-glycerol will be greatly reduced ($p < 0.05$). Meanwhile, more external ^{3}H-glycerol will exist in the kidney, fat, intestines, liver, brain and other tissues and organs. Injecting CNT can greatly change the quantity of ^{3}H-glycerol quantity in some tissues and organs ($p < 0.05$).

Other studies demonstrated that catecholamine, hyperglycemic factor, corticotrophin, growth hormone, hypertension, histaminase, luteinizing hormone, antidiuresis hormone and parathyroid gland hormone could help to increase the level of cAMP and help triglyceride to hydrolyze (Dodson et al., 1997). Whereas, insulin helps decreases the amount of cAMP and helps synthesize triglyceride. As the second messenger for most of nitrogenous hormone, growth factor and cytokine, CNT affect the liveliness and other activities of cell endoenzyme. In this way, hormones, growth factors and cell factors adjust the activity of cells. Meanwhile, the fat metabolism and the polarization and fat cells are also affected by these factors. This experiment demonstrates the same results as previous experiments did.

In this study, the bicameral model (the central and the peripheral) of $\hat{Y}_{(t)} = Ae^{-\alpha t} + Be^{-\beta t} - (A+B)e^{-kt}$ is optimized and then used as the dynamic model. The results show that the $\hat{Y}_{(t)} = Ae^{-\alpha t} + Be^{-\beta t} - Ae^{-\gamma t} - Be^{-\delta t} + C$ model fits well with this experiment when the medicine was injected through the belly skin. The examined central and peripheral tissues absorb the medicine at a different rate. A fixed amount of glycerin exists in the blood all the time. With additional constant (C) added into the model, the experiment has found out a better dynamics equation of: $\hat{Y}_{(t)} = 2850006.0485e^{-0.101002694t} + 10692.01131e^{-0.072195884t} - 2850006.0485e^{-0.100728077t} - 10692.01131e^{-0.170750988t} + 535.4090851$. The result shows that after injection of ^{3}H-glycerol, the concentration of ^{3}H-glycerol in the central and peripheral tissues reaches the highest point after 5.4704 h at 1,071.6095 dpm/mL with 99.62% exists in the central, before it transferred to the peripheral and the concentration between the 2 initially reaches a balanced point and then gradually decreased. ^{3}H-Glycerol accumulates in the blood fat of all the tissues and organs and can hardly be decomposed by the body in ordinary breeding environment. Therefore, the concentration of ^{3}H-glycerol cannot be reduced to zero. However, rabbits injected with cyclic nucleotides will have different hormones and enzymes. So the common metabolic regu-

larity does not apply to them, as indicated by the control group.

In conclusion, we established the particular tracing kinetic equation of metabolism of glycerol and were clear about the distribution of glycerol in Haerbin white rabbits in China. The present study provided a basis for further studies on the lipid metabolism and fatting of large individual animals.

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