The penetration enhancement and the lipolystic effects of TAT-GKH, in both *In vitro, Ex vivo,* and *In vivo.*

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Summary

It was demonstrated that Transactivating transcriptional activator(TAT) protein from HIV-1 shown to enter cells when added to the surrounding media. TAT peptide chemically attached to various proteins was able to deliver these proteins to various cell and even in tissues in mice with high levels in heart and spleen.

In this study, the tripeptide GKH(Glycine-Lysine-Histidine) derived from Parathyroid hormone (PTH), which was known as lipolytic peptide, is attached to 9-poly Lysine(TAT) to be used as a cosmetic ingredient for slimming products. When Glycerol release, expressed as extracellular glycerol concentration, is lipolysis index, TAT-GKH at 10^{-5}mol/L induces approximately 41.5% maximal lipolytic effects in epididymal adipocytes isolated from rats, compared with basal lipolysis. Epididymal adipose tissues of male rats is assessed *ex vivo* by microdialysis. Probes are perfused with Ringer solution in which increasing concentrations of TAT-GKH. The perfusion of TAT-GKH induces lipolytic effect. Penetration study showed that TAT-GKH efficiently elevates 36 times higher penetration into the excised hairless mice skin than GKH. *in vivo* study showed that TAT-GKH had a better effect upon the relative volume of eye bag after 28 days of application on twenty(+2) healthy female volunteers.

It was identified that TAT-GKH increases penetration enhancement and lipolytic effects in both *in vitro, ex vivo* and *in vivo*.

Introduction

In the effort of overcoming the limiting barrier of drug delivery to skin, various physical and chemical vehicles such as micelles, liposomes, microemulsions and nanopaticles have been used in cosmetic science as well as in pharmaceutical science. However, these approaches have been observed not only to be dependent on the applied constituents of the vehicle but also drastically on the composition/internal structure of the phase, which may hamper drug diffusion in the vehicles (1-3).

Recently, the use of peptide and protein domain with amphipatic sequences for drug and gene delivery is getting increasing attention (4). The basic domain of human immunodeficiency virus type I (HIV-1) transactivator of transcription (TAT) protein was reported to possess the ability to traverse the biological membranes efficiently in a process termed 'protein transduction' (5-7). Furthermore, TAT peptide chemically attached to various proteins including horseradish peroxidase, β-galactosidase and ovalbumin was able to deliver these proteins to various cells and even in mice tissues, with high levels in heart, lung and spleen (8,9). Although the actual mechanism of TAT has not yet clearly established, common structural features of TAT include the presence of many basic amino acids (arginine and lysine) as well as the ability to adopt an alpha helical conformation (7).

Parathyroid hormone (PTH) is a peptide hormone, a long chain containing 84 amino acids. Although its principal activity is calciotropic, published investigations have revealed that it also shows lipolytic activity in human adipose tissue (10,11). Recently, PTH derived tripeptide(GKH, Glycine-Lysine-Histidine) was reported to stimulated β-adrnergic receptor coupled to adenylyl cyclase via the stimulatory Gs protiein, increment of cAMP production which leads to activation of protein kinases A and phophorylation of hormone-sensitive lipase(HSL) resulting in glycerol release by fat cell (12).

This short fragment of GKH that is non permeable into the cell was successfully attached to TAT (9-polylysine) to have both the lipolytic and penetration effects into the skin, without affecting the lipolysis activity of GKH.

In this study, we investigated the possibility of TAT-GKH as delivery vehicles without the loss of lipolytic effect of GKH through TAT-GKH fusion, and discussed the usefulness as a cosmetic ingredient for the slimming products.

Materials and Methods

Preparation of TAT-GKH peptide

TAT-GKH peptide (KKKKKKKKK-GKH) was prepared with an automated peptide synthesizer (Applie Biosystem 433A) by using standard solid-phase fluoenylmethoxycarbonyl (Fmoc) chemistry with HATU as the peptide-coupling agent. Cleavage from the resin was achieved by using a mixture of trifluoroacetic acid (TFA / H_20 / ethanedithiol / phenol / thioanisole). Removal of the solvent gave a precipitant that was triturated with cold diethyl ether. The crude mixture obtained was centrifuged, then was removed by decantation, and the resulting orange solid was purified by HPLC (Shimazu LC-8A) in the linear gradient of 28-30% of CH_3CN in 0.1% TFA for 15 min. The product was isolated by lyophylization and characterised by MALDI-TOF mass spectrophotometer (Perseptive Biosystems Voyager Linear Mass Spectrophotometer) by using α-cyano-4-hydroxy-cinnamic acid as a matrix. The purity of the peptide was >95% as determined by analytical HPLC (13).

Animals

Male Sprague-Dawley rats weighing 370-420g and 8-9 weeks old female hairless mouse weighing 27-33g (DaeHan Biolink, Taejeon, Korea) were housed in a temperature-controlled

room (22±2°C), and subjected to a 12-hour light/dark cycle. Animals had free access to laboratory food and water and were carefully handled.

***in vitro* Lipolysis Studies**

For *in vitro* experiments rats were sacrificed by cervical dislocation after an overnight fast, and epididymal adipose tissue was immediately removed. Isolated fat cells were obtained by collagenase digestion (1 mg/mL, 37 °C) in Krebs-Ringer bicarbonate buffer, containing 3.5 g/100 mL of bovine serum albumin (BSA V) and 0.6 mM of glucose at pH 7.4 (KRBA), under continuous vigorous shaking (90 cycles/min) according to the method of Rodbell (14). Fat cells were filtered through nylon mesh and washed three times with the same incubation buffer (KRBA), to eliminate the stroma-vascular fraction and collagenase A. Measurements of lipolytic activity were performed by incubating isolated adipocytes in 200 $\mu\ell$ of KRBA buffer with continuous gentle shaking (30 cycles/min). After 2 hours of incubation with TAT-GKH (10^{-3} mol/L ~10^{-7} mol/L) at 37 °C, the reaction was stopped with ice and an aliquot (50 $\mu\ell$) was taken to determine glycerol release in the incubation buffer by the method of Wieland (15). Basal lipolysis was determined in the absence of TAT- GKH. The metabolic activity was expressed as micromoles of glycerol released per 100 mg of total lipids, which were determined gravimetrically after their extraction, according to the method of Dole and Meinertz (16).

***ex vivo* Lipolysis Studies by Microdialysis**

The day of experiment, after an overnight fast, the rats were anaesthetized with a subcutaneous injection of urethane (1.5g/kg body weight).

First, a 1.5cm skin incision in the abdominal region was made. After *obliquus internus abdominis* muscle incision, a microdialysis probe was inserted into the epididymal adipose tissue in each side, with the help of a steel-guide cannula. Correct implantation of the probes was ascertained by visual inspection. Body temperature during the experiment was maintained with an electric

blanket.

The dialysis probes consisted of a polycarbonate tubular membrane with a 0.5mm outer diameter, a 10mm length and a 20kd molecular weight cut-off (CMA/20 Carnegie Medicin, Stockholm, Sweden). Before use, the probes were perfused with Ringer solution, which effectively removed all glycerol in the membrane. The probes were connected to a microinjection pump (Harvard Apparatus) and perfused at 2.5$\mu\ell$/min with Ringer solution (154mmol/L sodium, 4mmol/L potassium, 2.5mmol/L calcium and 160mmol/L chloride) supplemented with ethanol (1g/L).

Immediately after implantation, each probe was continually perfused with the Ringer solution. At first, dialysis probes were rinsed for 30min to eliminate the transient adenosine triphosphate concentration due to the initial trauma of implantation. During this stabilization period no outgoing dialysate was collected. After that, 10-minute fractions were collected during 30 min and were referred to as the basal condition. Then, Each ringer solution containing four TAT-GKH concentrations (10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} mol/L) were used. At each concentration, there was a 40-minute perfusion and samples were collected at 10-minute intervals during 40min in order to provide a mean value. Glycerol concentration in each dialysate was measured as the index of lipolysis in 10 $\mu\ell$ samples with an ultrasensitive radiometric method (17).

Cytotoxicity Assay

Epididymal adipocyte isolated from rats were seeded in 96well plates at a density of $5x10^3$ cells/ml/well. The used same doses of TAT-GKH were added to the culture medium. The cytotoxicity was evaluated by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrzolium bromide] test (18) and measuring formazan formation spectrophotometically at 570nm.

Local blood flow during microdialysis

Blood flow changes around the microdialysis probe were measured using the method described

by Hickner et al. (19) using ethanol at 1g/L. Ethanol rapidly diffuses through the membrane probe. Hickner et al. showed that ethanol clearance from the perfusion medium is related to local blood flow. Changes in blood flow are presented as a percentage of baseline (outflow/inflow) x 100, that is, ethanol in the dialysate/ethanol in the perfusate x 100. Ethanol was determined with an enzymatic method in 5$\mu\ell$ samples of dialysate (20).

***ex vivo* Skin Permeation Studies**

Vertically assembled Franz type diffusion cells (Microette transdermal diffusion system, Hanson Research Corporation, Chatsworth, CA, USA) were used for in vitro skin permeation experiments. The system consisted of Franz type diffusion cells with an effective diffusion area of 1.776 cm^2 and receptor volume of 7.0 ml, autosampler, and cell drive system with rpm controller. The fundamental experiments were performed according to the method given in our previous report (21). Briefly, the excised skin of female hairless mouse was obtained from 8-9 weeks old, 27-33 g animals. The dermal side of skin was soaked in buffer with 15% ethanol solution containing 5mM phenylmethylsulfonyl fluoride (PMSF) for 12h at 10℃ to inhibit the enzyme. The skin was mounted on diffusion cell, and the receiver compartment was filled up with 7ml of 50mM PBS with 15% ethanol containing 5mM PMSF and maintained at 32℃ by circulating water within a jacket around the lower chamber. PMSF was used to inhibit the enzyme and 15% Ethanol was used to dissolve PMSF. The ethanol concentration had no effect on the penetration of peptides, as reported by Ghanem et al., who showed that ethanol at low levels (<25%) had little or no effect on pore pathway (22). 20% ethanol solution containing 1% TAT-GKH(w/v) and 1% GKH(w/v) were applied in the donor compartment. 20% ethanol solution was uniformly distributed with a micropipette on the skin surface (100$\mu\ell$). The receptor fluid was mixed by a magnetic stirrer throughout the experiment. The receptor fluid was collected from the receiver compartment at predetermined time (every 12 hours after sample application) and

replaced by fresh fluid.

At the end of the experiment (24 hour after sample application), receptor fluid was collected, and donor compartment was washed with 500㎕ of ethanol three times. After completion of the preset time (24 hour), skin samples were taken out of diffusion cells. The skin was homogenized by 4 ml of PBS to extract TAT-GKH and GKH. After filtration on Millex filter FG (pore size: 0.2㎛, millipore), solutions were assessed by HPLC. Five hundred microliters of receptor fluid withdrawn from the receiver compartment at predetermined times was treated with 10㎕ of TFA. Following centrifugation (13,000 rpm), the each amount of TAT-GKH and GKH in the supernatants was determined by analytical HPLC. The HPLC consisted of solvent delivery pump (Waters, 600 pump, Waters Co., MA, USA), C_{18} column (HP ODS Hypersil 5㎛, 4.6×150 mm, Hewlett Packerd, Germany), UV detector (waters, 486 UV detector) and data process system (Waters millennium). TAT-GKH was analyzed with the mobile phase of distilled water in 0.1 % TFA. TAT-GKH was analyzed with the mobile phase of 15% acetonitril in 0.1 % TFA and at the flow rate of 1 ml/min. The absorbance at 210nm was measured for the assay of those peptides. The retention time was 4.5 min for GKH. The retention time was 12.3 min for TAT-GKH. Temperature of the column was kept at 40℃.

Statistical Analysis

Values are given as means ±SEM of (n) separate experiments. Difference in value was statistically analyzed using a paired t-test.

***in vivo* Assessment**

Twenty(+2) healthy female subjects, aged between 30 and 55 years old, participated in this study for 28days. All the volunteers applied an emulsion containing TAT-GKH twice a day under the eyes. The other emulsion, which did not contain TAT-GKH as placebo were applied under their other eyes. The volunteers did not apply the emulsion on the day of measurements. The

results obtained at various assessment times with the technique of fringes projection were compared with those obtained at T0 day. The fringes projection system(halogen projector) was coupled with a CCD high resolution camera(black and white-768x512 pixels; field of view 30x210x270㎜3)(FaceScanner, Breuckman, Germany)-linked with the acquisition software Optocat(Breuckman, Germany). The lighting system, coupled to a video camera permits the projection of a sequence of line lattice onto the object (fringes). Three-dimensional information of the object is calculated from the deformation of these fringes on the surface of this one and recorded by the numeric camera. The significance of the results was determined using Student's t-test. This test was applied to the deviation in relation to T0 day.

Results

in vitro Lipolysis Studies

Due to the fusion of TAT into GKH, it was necessary to confirm whether TAT-GKH like GKH still had the same lipolytic effects or not. To compare the lipolytic effects of TAT-GKH with those of GKH, epididymal adipocytes isolated from rats were incubated in the presence and absence of various doses of TAT-GKH and of GKH at 37℃ for 2h. Lipolysis in the epididymal adipocityes was estimated by determining the amount of glycerol released into the medium as a result of lipolysis on triglyceride. Lipolytic agent isoproterenol was used as the positive control to confirm no technical errors in this experiment and induced high lipolytic effects (data not shown). TAT-GKH and GKH induced approximately 41.5% and 36.3% maximal lipolytic effects at 10^{-5} mol/L (0.77 ± 0.01 and 0.74 ± 0.04 glycerol μmol/ lipid mg/ h), respectively (Table. I., Fig.1). These results suggested that TAT-GKH was capable of inducing triglyceride breakdown in epididymal adipocytes. These results showed that the fusion of TAT into GKH do not significantly modified the its own lipolytic effect of GKH.

***ex vivo* Lipolysis Studies by Microdialysis**

The effect of increasing concentrations of TAT-GKH on glycerol production in adipose tissues is depicted in Table II. The addition of 10^{-3} mol/L and 10^{-4} mol/L TAT- GKH induced a statistically significant increase in glycerol concentration (55.9% and 73.9% respectively). No significant difference in glycerol concentration were seen in lower TAT- GKH concentrations (10^{-6}~10^{-5}mol/L) against the basal condition.

Cytotoxicity on Adipocytes

To determine whether the lipolytic effects of TAT-GKH in the concentration of 10^{-4} and 10^{-3} mol/L TAT- GKH were induced by the cytotoxicity, and the applicable possibility as the cosmetic ingredient for slimming products or not, we measured the cell viability with MTT tests, which evaluates mitochondria integrity. TAT-GKH produces no cytotoxicity at any dose concentration (Fig. 2). This result showed that the TAT-GKH not only induced lipolytic effects by it's own activity, but also could be used as cosmetic ingredient for the slimming products in safety.

Effects on Local blood flow

Local blood flow around the probes was unchanged during the microdialysis experiment (data not shown).

***ex vivo* Skin Permeation Studies**

To identify the skin permeability of TAT-GKH in excised hairless mouse skin, vertically assembled Franz type diffusion cells were used. Table III shows the permeated amounts of TAT-GKH in 20% Ethanol solution 24 hours after application to the excised hairless mouse skin. The permeated amount of TAT-GKH was 36 times greater than that of GKH. It was considered that this result was due to increase in partition of GKH to skin by TAT fusion.

***in vivo* Assessment**

Prior to the application of the test product, no significant difference in the relative volume was

observed between the relative volume of eye bags treated by the TAT-GKH containing emulsion and the relative volume of eye bags treated by the placebo emulsion($P>0.05$). The mean results for the relative volume of eye bags for the test panel as a whole are presented in table Ⅳ. A reduction in the relative volume of eye bags was noted on T+28 days(-2.33 mm^3) after application of the TAT-GKH containing product. This variation corresponds to a 6.4% reduction that is statistically significant ($p<0.05$). After application of the placebo product, the relative volume of eye bags also decrease(-1.09mm^3). This variation corresponds to a 2.8% reduction that is not significant. No significant difference between the relative volume of eye bags treated by the TAT-GKH product and the relative volume of eye bags treated by placebo has been observed for the values related to T0day.

Dicussion

A variety of materials in cosmetic business have been attempted to achieve local fat reduction over many years. These attempts have not been shown to be effective. One of these reasons is that potential lipolytic materials cannot efficiently penetrate the skin barrier. The peptide of TAT-GKH with bi-functional effects (skin penetration by TAT(9-polylysine) and lipolysis by GKH) was synthesized to use as a cosmetic ingredient for slimming products. TAT-GKH at the 10^{-5} mol/L concentration showed maximal 41.5% lipolytic effects in isolated adipocytes from the rat, without modifying previously reported lipolytic effects of GKH. In Ex Vivo studies, we just identified the lipolytic effects at the 10times higher concentration than in in vitro experiment. These lipolytic effects did not induced by the toxicity of TAT-GKH. When microdyalisis technique for lipolysis is used, it seems essential to assess changes in local blood flow to distinguish between events at the fat cell and at microcirculation. The extracellular space is influenced not only by fat cell metabolism, but also by microcirculation. Thus, the concentration of a particular

substance in the extracellular space depends on blood flow and on the metabolism by fat cells (23,24). In order to study the potential effects of TAT-GKH perfusion on local blood flow, ethanol was added to the microdialysis perfusion medium. As ethanol readily diffuses through the probe membrane and as it is not metabolized by adipose tissue (25), its escape from the perfusion medium to the extracellular compartment, measured by the ethanol outflow/ethanol inflow ratio, would be dependent on blood flow in the tissue surrounding the microdialysis probe (26).

In ex vivo skin permeation studies, TAT-GKH elevated 36 times higher penetration into the mice skin than GKH. We had challenged TAT-GKH in mice to obtain antibody against TAT-GKH to identify the penetration of TAT-GKH into skin through immunohistochemistry, but it failed to raise the antibody against TAT-GKH in mice. But there is an indirect evidence that TAT(9-polylysine)-SOD (TAT-Superoxide dismutase) efficiently penetrated into the epidermis as well as the dermis, when topical applied an the mice skin, as judged by immunohistochemistry and specific enzyme activities (9).

In vivo studies demonstrate that 6.4% fat reduction as measured by eye bags, can be produced by topical application of the TAT-GKH containing emulsion. Even though we did not directly measure the fat loss in eye bags, the technique of fringes projection, adapted to the analysis of the eye bags allows to objectify the decrease of these eye bags after the topical treatment. This technique allows the direct and immediate acquisition of the morphology of the topical applied area in 3 dimensions.

Conclusions

The peptide of TAT-GKH with bi-functional effects (skin penetration by TAT(9-polylysine) and lipolysis by GKH) was synthesized to use as a cosmetic ingredient in slimming products. In vitro studies showed that TAT-GKH at 10^{-5}mol/L induces approximately 41.5% maximal lipolytic

effects in epididymal adipocytes isolated from rats, compared with basal lipolysis. We reconfirm the lipolytic effects of TAT-GKH in *ex vivo* with the microdialysis. There was no cytotoxicity in any dose concentration of TAT-GKH. In addition, TAT-GKH elevated 36times higher penetration into the mice skin than GKH. Our result showed that the small peptide of TAT-GKH induced the lipolytic effects and efficiently increased penetration into mice skin. Twenty(+2) healthy female subjects had 6.4% fat reduction on their eye bags by topical application of the TAT-GKH containing emulsion. This study suggested that TAT-GKH could be used as a cosmetic ingredient for slimming products

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Table I. Maximal lipolytic effects of TAT-GKH in epididymal adipocytes from rats compared with those of GKH and isoproterenol.

Lipolytic agents	Glycerol μmol/ lipid mg/ h
Basal lipolysis	0.54 ± 0.01
Isoproterenol (10^{-6}mol/L)	5.74 ± 0.64
GKH (10^{-5}mol/L)	0.74 ± 0.04**
TAT-GKH (10^{-5}mol/L)	0.77 ± 0.01**

Values are mean ±SEM(n=6) and are expressed as Glycerol μmol/ lipid mg/h. **P<0.01 when compared to values obtained with basal lipolysis.

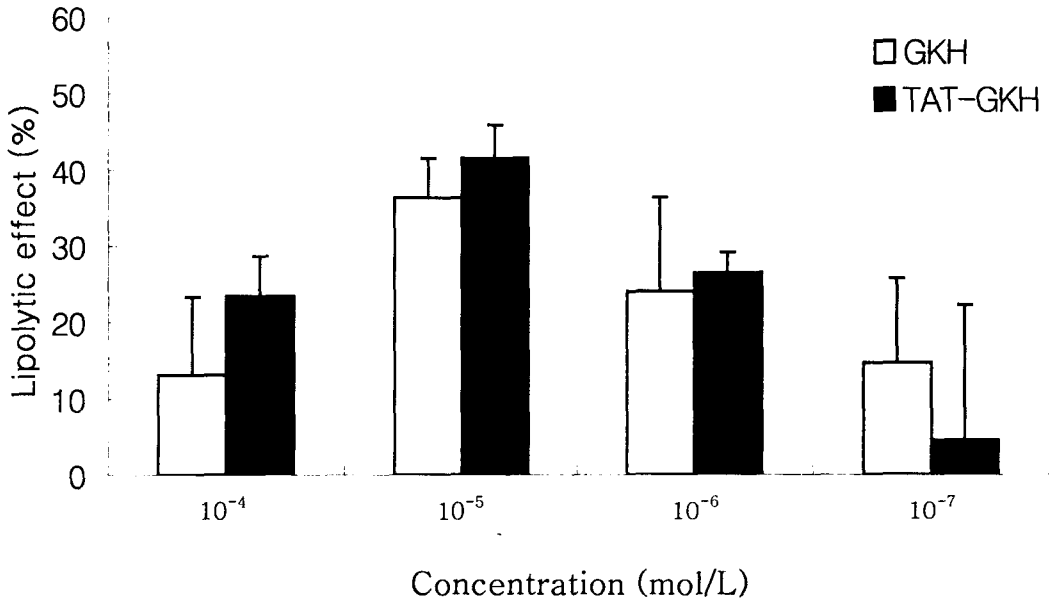

Figure 1. Lipolytic effects of TAT-GKH in epididymal adipocytes from the rats compared with those of GKH. Values are mean ±SEM(n=6), expressed as percent(%) vs basal lipolysis for glycerol and significantly different from basal lipolysis.

Table II. *Ex vivo* lipolytic effect of increasing concentrations of TAT-GKH in epididymal adipose tissue.

	TAT-GKH(10^{-3} mol/L)	TAT-GKH(10^{-4} mol/L)
Basal lipolysis (μmol/L)	48.33 ± 19.6	62.67 ± 10.8
TAT-GKH lipolysis (μmol/L)	75.33 ± 9.33*	109.0 ± 19.7*
Lipolytic effect	55.9 %	73.9 %

Values are mean ±SEM(n=3) ***P<0.05,** when compared to values obtained with basal lipolytic effect.

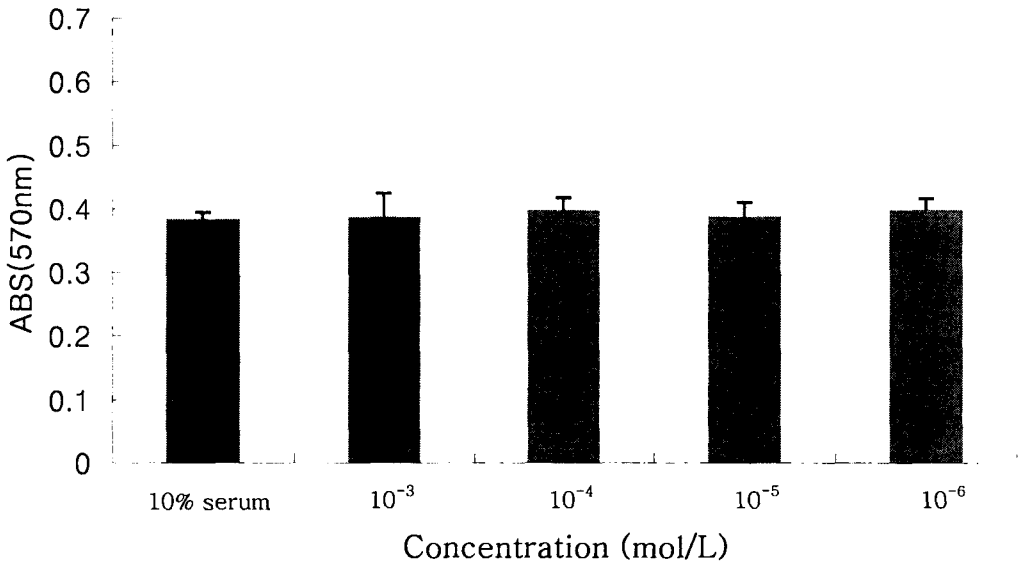

Figure 2. Cytotoxic effects of TAT-GKH at dose-concentrations on epididymal adipocytes through MTT tests. Values are mean ±SEM(n=6) and are expressed as abrsoption(ABS) at 570nm.

Table III. Total Permeated Amount (%) of TAT-GKH the Excised Hairless Mouse , 24 Hours After Application.

	GKH (μg)	TAT-GKH (μg)
Mice skin	2.73 ± 0.22	119.66 ± 9.33***
Receptor Solution	1.60 ± 0.13	36.67 ± 4.85***
Total permeated amount	4.34 ± 0.35	156.34 ± 9.02***
%	0.43 %	15.63 %

Values are mean ±SEM(n=6) ***P<0.001 when compared to values obtained with GKH

Table Ⅳ. Mean variation in the relative volume of the bottom part of the face treated by Placebo cream and TAT-GKH containing cream.

	Placebo cream	TAT-GKH containing cream
T + 0 day (mm^3)	38.57 ± 14.2	36.13 ± 15.9
T + 28 day (mm^3)	37.94 ± 12.9	32.50 ± 15.2
Evolution of the volume(%)	- 1.09%	- 6.4%

T + 0 day = baseline before treatment.

T + 28 day = 28days after the beginning of the treatment.

Relative volume : volume between the region of interest defined on eye bags and the average plane of matched acquisition(T0 and T28).