Mortazavi et al., *BioImpacts*. 2025;15:30071
doi: 10.34172/bi.30071
https://bi.tbzmed.ac.ir/

Topically applied GHK as an anti-wrinkle peptide: Advantages, problems and prospective

Seyedeh Maryam Mortazavi[1], Seyyed Ali Mohammadi Vadoud[2], Hamid Reza Moghimi[1,3*]

[1]Department of Pharmaceutics and Pharmaceutical Nanotechnology, School of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[2]Department of Pharmaceutical Biotechnology, School of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[3]Protein Technology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Article Info

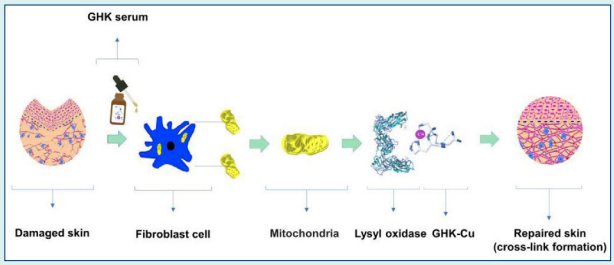
Article Type:
Review

Article History:
Received: 26 Aug. 2023
Revised: 26 Oct. 2023
Accepted: 31 Oct. 2023
ePublished: 28 Apr. 2024

Keywords:
GHK
GHK-Cu
Pal-GHK
Anti-wrinkle peptides
Skin permeability
Cosmetic peptides

Abstract

Introduction: Peptides are promising and attractive anti-wrinkle active ingredients, amongst which glycyl-histidyl-lysine peptide (GHK) is one of the most broadly promoted peptide for topical application. This simple sequence of amino acid residues not only has the capability of tissue regeneration and the enhancement of collagen and glycosaminoglycans synthesis but also is able to increase nerve outgrowth and angiogenesis. Consequently, GHK has several properties, from wound healing to prevention/reduction wrinkles. GHK-Cu and Pal-GHK are metal complex and palmitoylated derivatives of GHK, respectively. Although GHK-Cu and Pal-GHK are widely used in anti-wrinkle products available on the cosmetic market, the published information on their skin permeability, effectiveness, physicochemical properties and so on is insufficient.



Methods: This review aims to highlight whether GHK is sufficiently effective on wrinkle prevention/reduction. Apart from the effectiveness, another question that is tried to be answered is whether skin permeability of GHK allows it to act as an anti-wrinkle peptide at its site of action? Skin permeation enhancement methods employed so far are also reviewed.

Results: Based on cellular studies, undoubtedly, GHK can be considered as an anti-wrinkle ingredient. Although GHK-Cu and Pal-GHK have been of interest as effective peptides to be incorporated in the anti-wrinkle products, there is a surprising absence of clinical studies using them. Metal complexation and chemical modification with a hydrophobic moiety increase permeability of this peptide. Besides, cell penetrating peptides seem promising to increase skin permeation of GHK and its derivatives. Skin pretreatment with microneedles also has the potential to be further studied for permeation enhancement of such peptides. As peptide ingredients, their formulation may encounter some challenges, mainly due to their hydrophilic (high aqueous solubility and low partition coefficient) and unstable nature.

Conclusion: Although GHK-Cu and Pal-GHK are effective and relatively skin permeable, their permeability could be successfully increased using permeation enhancement methodologies.

Introduction

There are growing evidences that suggest the functional role of peptides in several areas, including in counteracting the effects of skin aging. Currently, peptides are becoming interesting anti-wrinkle cosmetic ingredients[1,2] of which glycyl-histidyl-lysine peptide (GHK) is a well-known peptide not only in the market of anti-aging products but also in the other aspects of sciences. There are four main groups for topical peptides, including signal peptides, carrier peptides, enzyme-inhibitor peptides and neurotransmitter-inhibitor peptides.[3] GHK is a signal peptide and capable of promoting the production of elastin, proteoglycans and glycosaminoglycans as well as regular collagen synthesis in the intact skin. In addition, GHK is a carrier peptide and able to deliver the important trace element copper into skin cells.[4,5] Two main available derivatives of GHK are copper peptide (GHK-Cu) and palmitoylated peptide (Pal-GHK) (Fig. 1).

GHK-Cu (Fig. 1) is a metal complex of GHK tripeptide with copper (II) ion, which forms spontaneously.

*Corresponding author: Hamid Reza Moghimi, Emails: hrmoghimi@sbmu.ac.ir; hrmoghimi@yahoo.com

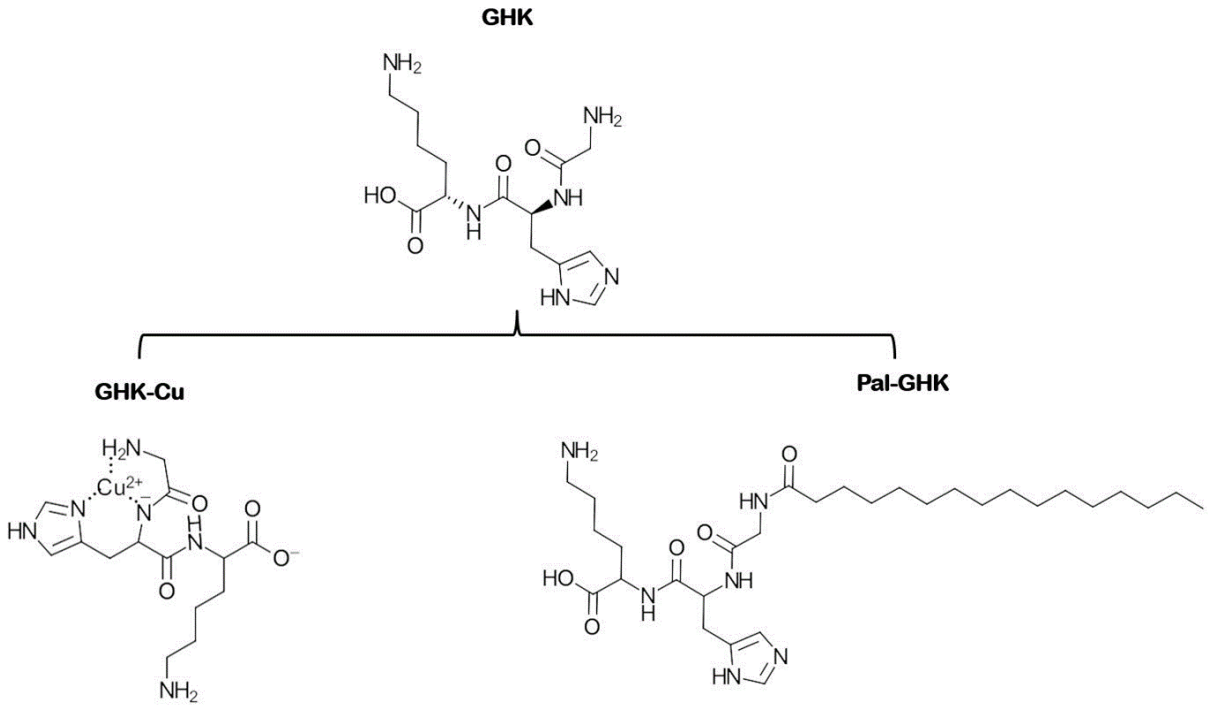


Fig. 1. Chemical structures of GHK and its copper and palmitoylated (Pal) derivatives.

According to X-ray analysis, glycine and histidine residues bind as a ligand to a metal cation (Cu^{2+}).[6] The lysine residual side chain may be involved in identifying receptors on target skin cells. As explained later, the involvement in the enzymatic anti-aging process is what the copper element does.[7] Both moieties of GHK-Cu, whether GHK or Cu^{2+}, play a role in preventing the aging process.

Palmitoyl-GHK (Pal-GHK, also known as palmitoyl tripeptide-1) is a derivative of GHK that is produced through the chemical modification using palmitic acid, a 16-carbon fatty acid (Fig. 1). The covalent attachment of palmitic acid, as a hydrophobic moiety to topical peptides is performed with the purposes of improving the skin permeability of peptides. Matrixyl™ 3000 is a combination of Pal-GHK and Pal-GQPR (glycine-glutamine-proline-arginine) which is commercialized as an anti-wrinkle product.[5]

Here, the bioactivity and effectiveness of GHK and its derivatives are initially reviewed and then the possibility of skin permeation of GHK, GHK-Cu and Pal-GHK and enhancement approaches are discussed.

Bioactivity of GHK

GHK is a leader sequence which improves the fabrication of extracellular matrix components including collagen, elastin and glycoproteins.[5] This peptide sequence proteolytically separates from its parent molecule, SPARC glycoprotein (Secreted Protein Acidic and Rich in Cysteine), one of the main secretory products of fibroblasts.[8]

As mentioned earlier, GHK can carry and transfer copper, a crucial element for the performance of the enzyme lysyl oxidase that is produced in the mitochondria of fibroblast cells. This essential enzyme for the stabilization of extracellular matrixes mediates cross-linking in connective tissue via catalyzing the oxidative deamination of amine group in lysyl residues in tropocollagen and tropoelastin. Lack of copper can lead to the lysyl oxidase insufficiency and subsequently mechanical changes in the skin.[7,9]

GHK peptide has multiple effects on biological and cellular pathways. New gene analysis data has shown that GHK/GHK-Cu affects a wide range of genes involved in the organism's response to stress, such as inflammation, pain, cancer, anxiety, injury and so forth. Incubation of human skin fibroblasts with GHK-Cu increased production of connective tissue elements (e.g., elastin and collagen) as well as increased the expression of tissue inhibitors of metalloproteinases in the skin.[10]

Integrins are a family of transmembrane adhesive receptors present on keratinocytes. These principal receptors bind to the extracellular matrix's (ECM's) ligands (e.g., fibronectin, vitronectin, collagen and laminin) and mediate cell-cell and cell- ECM adhesion.[11] Using cultured keratinocytes and skin equivalent models, the effects of GHK-Cu on keratinocytes were evaluated by Kang et al[12] which showed that the cell exposure to GHK-Cu led to stimulation of basal epidermal cells and subsequently increased integrin expression.

In addition to the cellular studies, the results of some animal studies have also confirmed that GHK-Cu has

beneficial effects on the skin. Maquart et al evaluated the effects of GHK-Cu on stimulation of connective tissue accumulation in the Sprague-Dawley rats. The sequential injections of various concentrations of GHK-Cu into the implanted wound chamber increased the dry weight, total protein, collagen, DNA and glycosaminoglycan content in the rat skin. This increasing effect was dose-dependent.[13]

Some studies involving human volunteers have also established the ant-wrinkle effects of GHK-Cu. Abdulghani et al[14] studied the effects of topical application of GHK-Cu, vitamin C, melatonin and tretinoin on the ultrastructure of skin. Twenty persons were included in this study for a time period of 1 month. The first group of ten used a topical GHK-Cu cream and a topical melatonin cream on the left and right thighs, respectively. The rest used vitamin C topical cream and retinoic acid topical cream on the left and right thighs, respectively. A comparison of skin biopsies at baseline and after one month of treatment revealed that GHK-Cu was the most effective treatment, so that procollagen synthesis by dermal papillary fibroblasts increased in 70% of volunteers treated with GHK-Cu, 50% treated with the vitamin C, and 40% treated with retinoic acid.[14]

Given the published studies, GHK-Cu is effective on improvement of the signs of photoaging. A GHK-Cu cream was applied to the area around the eyes of 41 female participants with mild to advanced photodamage for 12 weeks. This intervention displayed a better performance comparing to placebo or a vitamin K cream so that GHK-Cu eye cream improved appearance via reducing lines and wrinkles as well as increasing the skin thickness and density.[15] Besides the photoaged skin around the eye, GHK-Cu cream also affected the facial skin of female participants (n=71) with mild to advanced signs of photoaging. The application of GHK-Cu cream for 12 weeks caused the enhancement of skin density and thickness and the reduction of skin laxity, fine lines and the depth of wrinkles.[16]

No cellular or clinical studies were found about the evaluation of anti-wrinkle properties of Pal-GHK in the literature. Although the results of published cellular studies conducted on the other chemically modified anti-wrinkle peptides using such moieties have shown that the conjugation does not eliminate the bioactivity of anti-wrinkle peptides,[17,18] whether conjugation of a hydrophobic moiety to GHK alters its bioactivity is a question that needs to be answered experimentally.

Skin permeability of GHK, Pal-GHK and GHK-Cu

Based on the studies mentioned in the previous section, undoubtedly GHK is effective on wrinkle reduction. Although bioactivity is a required property for an anti-wrinkle peptide, it is obvious that in in-vivo conditions, skin permeability is a prerequisite to efficiency.[5]

GHK is a tripeptide with molecular weight (MW) of 340.4 Da and calculated logarithm of partition coefficient (clogP) of -2.24.[19] Although the MW of GHK peptide does not exceed the ideal MW cutoff of skin permeation (i.e., about 500 Da), its logP is not in the appropriate range (i.e., 0 to 4) for skin permeation. Besides, GHK is a charged molecule at physiological pH. These latter two properties restrict the permeability of GHK through the stratum corneum, the main skin barrier against peptides permeation. If GHK molecules can permeate the skin, they will expose to the proteolytic enzymes which is another obstacle for GHK reaching its target in the right concentration.

One approach to enhance the skin permeation of hydrophilic peptides is chemical modification using hydrophobic moieties. The chemical modification can simultaneously enhance the resistance of peptides against skin proteases. The covalent attachment of palmitic acid to the lysine residue of GHK converts it to a peptide conjugate (Pal-GHK) with MW of 578.8 Da and clogP of 1.14.[19] Due to the more hydrophobic nature of Pal-GHK, the affinity of this peptide to the intercellular lipid matrix of the stratum corneum is expected to be higher than that of GHK (clogP: -2.24). Although the MW of GHK is increased through chemical modification, the MW of this conjugate is not still far away from the ideal MW cut-off of skin permeation.

Another approach to increase skin permeation of peptides is metal complexation.[20] How metal complexation increases the skin permeability of peptides is still under question. GHK-Cu is a metal complex with the MW of 402.9 Da and clogP of -0.94. Using flow-through diffusion cells, Hostynek et al,[21] evaluated the permeation of GHK-Cu in human stratum corneum (SC), entire epidermis (SC + viable epidermis) and split thickness skin (entire epidermis+ a portion of dermis). Based on the results obtained using inductively coupled plasma mass spectrometry, the skin permeability coefficients (Kp) for GHK-Cu over a 48-hour study ranged from 3×10^{-7} cm/h across heat-separated epidermis to 5.5×10^{-3} cm/h across isolated stratum corneum. When the epidermis was used as membrane, the percentages of copper measured in the receptor phase was 0.006% of the applied dose (the least permeated percentage). In the case of the SC and split thickness skin, 20% and 2% of applied dose were found in the receptor phase, respectively. Due to the absence of underlying skin layers beneath the isolated SC which retain the permeation of copper peptide, such high level of penetration across SC was observed. The most (438-fold increase over baseline) and the least (31-fold increase over baseline) retention belonged to the SC and split thickness skin, respectively. Since GHK-Cu is a complex, such high membrane retention in the stratum corneum was attributed to the intradermal decomplexation of GHK-Cu and re-binding of copper to endogenous ligands (e.g., amino acids) which possess higher nucleophilic donor

capacity compared to GHK.[21]

In a comparative study, Parke et al, evaluated the permeability of GHK, GHK-Cu, and Pal-GHK through a synthetic epidermis (Neoderm®-E).[22] Franz diffusion cells with the receptor phase of phosphate buffered saline were used in a 24-hour permeability study. As expected, the most and the least permeation belonged to the Pal-GHK and GHK, respectively. The percentages of cumulative permeated peptide were in the following order: Pal-GHK (4.61%) > GHK-Cu (3.86%) > GHK (2.53%). Based on the results of this study, the permeability of both derivatives is higher than that of parent peptide. The permeability of Pal-GHK is the most, indicating the more effectiveness of chemical modification with hydrophobic moiety compared with metal complexation. As mentioned earlier, the copper element is involved in the anti-aging process. GHK peptide is also an anti-wrinkle peptide. The complexation of two compounds with anti-wrinkle properties is ideal itself, especially when this complexation is able to increase skin permeability. However, much more studies (especially mechanistic studies) are required to answer the question of whether and how copper ions can increase GHK skin permeation.

GHK-Cu and Pal-GHK are used as active ingredients in many anti-wrinkle products available on the cosmetic market so that Pal-GHK was found as the second most used anti-wrinkle peptide in 2018,[23] however, not much studies are published on their skin permeability. Thus, further investigations focusing on human skin permeability of these derivatives are considerably required.

Permeation enhancement approaches for GHK, Pal-GHK and GHK-Cu

There are various chemical and physical approaches to increase the skin permeation of compounds. The chemical modification with hydrophobic moieties and metal complexation have been employed to increase the skin permeability of GHK peptide. However, to further increase the skin permeability of these derivatives as much as possible, some chemical and physical methods have also been applied. To the best of our knowledge, among all enhancement methods, only the effects of chemical modification with cell penetrating peptides (CPPs)[22] and pretreatment of skin with microneedles[24] have been evaluated in the case of GHK-Cu and Pal-GHK, as discussed below.

Topical peptides can be chemically modified with CPPs. These molecules work differently from hydrophobic moieties. CPPs are a sequence of 5 to 30 amino acids which are mostly polycationic. CPPs are capable of delivering small molecules and macromolecules to their targets inside the cells or inside the skin.[25-27] The skin permeability enhancement mechanism of CPPs has not been fully understood yet and most published studies are about how CPPs can increase permeability of cargos through vital cell membrane and not through the SC (composing of dead cells). However, based on the results of a few studies performed in this respect, CPPs affect both intercellular lipids and keratinized cells of the SC.[28] Arginine oligomers [R4(tetra-D-arginine), R6(hexa-D-arginine)] as CPPs have been chemically conjugated to GHK (Fig. 2), GHK-Cu and Pal-GHK and permeation of these conjugates were evaluated using a synthetic epidermis (Neoderm®-E) and Franz diffusion cells.[22] Both CPPs increased the permeability of the mentioned peptides, but R4 had a better performance than R6 (maybe because of smaller size). The increased permeability followed the order of GHK+R4 (7.6%) < GHK-Cu+R4 (8.7%) < Pal-GHK+R4 (9.8%).[22] These results might show that CPPs are suitable candidates for increasing skin permeability of anti-wrinkle peptides, however, further studies are required to elucidate all aspects of such potentials.

Microneedle pretreatment has been shown to be an efficacious approach to improve skin permeation of peptides or macromolecules such as proteins.[29-31] Skin pretreatment using solid microneedles create some microchannels in the skin which could be a potential delivery route for compounds.[32] Considering this capability, Li et al ascertained the effects of skin pretreatment using polymeric microneedle array on GHK-Cu permeability.[24] Using the 3M™ Microchannel Skin System (13 by 27 array, needle height: 700 μm, needle spacing: 500 μm, shape: square pyramidal) as well as vertical Franz diffusion cells (donor phase: 2 ml of 5.8 mM $[GHK]_2Cu$ solution, receptor phase: phosphate buffered saline, test duration: 9 hours) the permeation of GHK and GHK-Cu through human dermatomed skin was investigated. Also, the effect of application forces on depth and percentage of microneedle penetration was studied. The results showed that the higher the applied force the higher the depth and percentage of penetration. In the case of intact human skin (i.e., no pretreatment), no peptide or copper permeated, whereas about 134 nanomoles of GHK and 705 nanomoles of Cu permeated across the microneedle-pretreated skin. The use of microneedle pretreatment may be a useful approach to enhance skin permeation of such peptides.

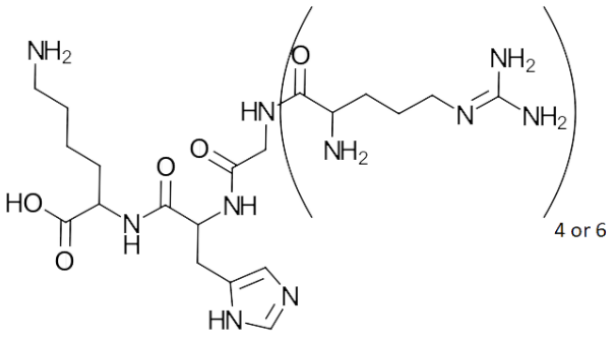


Fig. 2. The chemical structure of GHK+R4/GHK+R6.

Other suggestions for enhanced delivery of GHK

GHK and its derivatives are promising anti-wrinkle ingredients, so the improvement of their skin permeability can pave the way to more effective anti-wrinkle products. Although there are several methods to increase permeability of peptides, here, we intend to outline some of the applicable enhancement approaches that have been used successfully for other anti-wrinkle peptides. These approaches deserve to be given special attention for enhancing skin permeation of GHK. The MW and clogP values of all mentioned peptides in this section are presented in Table 1.

Chemical modification with hydrophobic moieties is a well-known approach to increase the skin permeability of anti-wrinkle peptides. Palmitic acid, as a hydrophobic moiety, was the first choice of researchers, so that peptide conjugates such as Pal-GHK, Pal-KTTKS (lysine-threonine-threonine-lysine-serine) and Pal-GQPR have successfully been synthesized and commercialized.[5] Other chemical permeation enhancers like terpenes have also been shown to have the potential to play the role of hydrophobic moieties. Terpene conjugation as a novel approach to improve topical delivery of anti-wrinkle peptides was introduced by our research team for the first time.[33] Two terpenes including citronellic acid and perillic acid with different chemical structures (linear and cyclic, respectively) were chemically attached to KTTKS, a signal peptide. Using human epidermis and a hydrophobic membrane model, the permeability of KTTKS, Pal-KTTKS, citronellic acid-KTTKS and perillic acid-KTTKS (see Table 1 for MW and clogP values) were evaluated. Contrary to KTTKS and perillic-acid-KTTKS which did not pass through the membranes, Pal-KTTKS and citronellic acid-KTTKS penetrated into both model and epidermal membranes, except that Pal-KTTKS was not found in the receptor phase. Since palmitic acid, a linear fatty acid, is a component of stratum corneum intercellular lamellar structure, and because of linear structure of KTTKS, the entrapment of Pal-KTTKS in the stratum corneum seems reasonable. The higher ability for diffusion through membranes (due to lower MW compared to that of Pal-KTTKS) as well as the lower chance for entrapment inside stratum corneum (due to having a ring in the structure of terpene) were proposed as reasons for higher permeation of citronellic acid-KTTKS.[33] Based on these results, the chemical modification of GHK using terpenes could be a new idea to enhance skin permeability of this peptide.

Table 1. The MW and clog P values of some anti-wrinkle peptides

Peptides	Molecular weight (Da)	clogP*
GHK	340.4	-2.24
Pal-GHK	578.8	1.14
GHK-Cu	402.9	-0.94
KTTKS	563.6	-3.45
Pal-KTTKS	802.1	3.72
Citronellic acid-KTTKS	715.9	-0.08
Perillic acid-KTTKS	711.8	-0.88
KT	247.3	-2.00
GQPR	456.5	-3.21
VGVAPG	498.6	-0.38
Acetyl hexapeptide-3	889.0	-6.67
Melanostatin	284.3	-1.07
GEKG	389.4	-2.58
Carnosine	226.2	-2.17

*All clog P values were calculated by ACD/ChemSketch freeware software.[19]

GHK: glycyl-histidyl-lysine, Pal: palmitic acid, KTTKS: lysine-threonine-threonine-lysine-serine, KT: lysine-threonine, GQPR: glycine-glutamine-proline-arginine, VGVAPG: valine-glycine-valine-alanine-proline-glycine, GEKG: glycine-glutamic acid-lysine-glycine.

Although chemical modification of hydrophilic peptides with palmitic acid creates a conjugate with more hydrophobic nature, the increased MW of the conjugates in comparison to the parent peptide causes a new problem for permeation through skin. Therefore, using fatty acids with shorter chain length as hydrophobic moiety could be brought up an applicable suggestion for GHK permeability improvement.[34] In another investigation of our group, fatty acids with different carbon chain lengths (C8, C10, C12) were chemically attached to the anti-wrinkle dipeptide KT (lysine-threonine). C8-KT with the lowest molecular weight showed the most skin permeation.[35]

Besides adding hydrophobic moieties to the peptides, the structures of anti-wrinkle peptides themselves can also be modified. For example, in order to decrease the formation of zwitterionic form, the side chains of amino acids residues of acetyl-hexapeptide-3 were chemically modified and it has been shown that this strategy affects the skin permeability of this peptide positively.[36]

Skin pretreatment with chemical penetration enhancers or using these substances in the formulation of cosmetic peptides has been shown as a relatively efficacious enhancement approach. Despite the simplicity of implementation, this method has not yet been investigated to increase GHK skin permeability. However, 1,2-pentylene glycol, an amphiphilic diol, was demonstrated as an effective enhancer for carnosine, an antioxidant dipeptide.[37] Besides, propylene glycol has shown the ability for enhancing skin permeability of the acetyl-hexapeptide-3 (Argirelin®). As a neurotransmitter inhibitor peptide, acetyl-hexapeptide-3 affects the chemical synapse by inhibiting the release of acetylcholine.[36,38] We did not find any publication on the effects of other chemical penetration enhancers (e.g., amides, fatty acids, surfactants, essential oils, terpenes, esters and so forth) on skin permeability of anti-wrinkle peptides. To some up, chemical penetration enhancers (either physical mixture

with peptides or chemically attached to peptides) seem to be good candidates to improve skin permeability of GHK and its derivative.

In addition to chemical strategies, various physical enhancement methods are employed to affect skin permeability of small molecules and macro-molecules but when it comes to the anti-wrinkle peptides, an obvious paucity of literature is observed. Microneedle is a more common physical strategy to enhance delivery of anti-wrinkle peptides so that the delivery of GHK-Cu,[24] Pal-KTTKS, melanostatin,[39] GQPR, acetyl hexapeptide-3 and VGVAPG (valine-glycine-valine-alanine-proline-glycine)[40] has been investigated after microneedle treatment. Another physical method applied for anti-wrinkle peptides is iontophoresis. The iontophoretic delivery of acetyl-hexapeptide-3 through skin has been evaluated. Comparison with passive permeation, the iontophoresis at pH 7.4 caused a 30-fold increase in the skin permeability of this peptide. Due to the net zero charge of acetyl-hexapeptide-3 at the applied pH, the electroosmosis was considered as the main permeation enhancement mechanism.[41] It is highly suggested to investigate the effects of iontophoresis and other physical strategies on skin permeation of anti-wrinkle peptides, specially GHK and its derivatives.

Various types of nanostructures are being investigated in the field of cosmetic as active agents (e.g. nanometals) or carriers (e.g. liposomes).[42] Anti-wrinkle peptides can also benefit encapsulation into nanocarriers since these carriers not only can increase skin permeation and retention but also protect the cargo from destabilizing agents inside the barrier. In the case of anti-wrinkle peptides, amongst numerous carriers used for delivery of cargos, lipid-based carriers such as phospholipid-based vesicular nanocarriers, liquid crystalline nanoparticles and microemulsions have been received particular attention. The phospholipid-based vesicular nanocarriers including transformer-ethosomes, ethosomes and liposomes have been loaded by Pal-KTTKS and compared with each other in terms of flexibility and ability to enhance skin permeation of the cargo.[43] The transformer-ethosomes with the size range of 77.2 nm to 112.2 nm not only exhibited the most flexibility but also performed better than ethosomes (particle size: 99.7 nm) and liposomes (particle size: 112.2 nm) in terms of permeability enhancement so that in permeation study test, only Pal-KTTKS loaded in this type of carrier were found in the receptor phase. Encapsulation of Pal-KTTKS into liquid crystal nanoparticles also resulted in the enhancement of both skin permeability and retention.[44]

To act as an anti-wrinkle peptide, GEKG (glycine-glutamic acid-lysine-glycine, MW: 389.4, clog P: -2.58) should reach to its target in the skin i.e., the dermis layer. This tetrapeptide is a collagen synthesis stimulator. A W/O microemulsion congaing GEKG were prepared and its skin permeation compared with that of a standard cream. Results have shown that the remnant peptide in the stratum corneum following microemulsion application was much lower than standard cream, while the microemulsion has allowed much higher amount of GEKG to meet deeper skin layers, the targets.[45]

In the case of GHK, GHK-Cu and Pal-GHK, to the best of our knowledge, this is not much data on the application of nano-carriers toward skin delivery of these peptides. Only in one study[7] and in order to assess the compatibility of GHK-Cu with common components of lipid-based nanocarriers, niosomes composed of cholesterol and sorbitan monostearate with and without dicetyl phosphate, the surface charge modifying surfactant, have been used.[7] GHK-Cu loaded niosomes composed of sorbitan monostearate and cholesterol exhibited no degradation after being stored at 40°C for 1 month. On the contrary, GHK-Cu loaded niosomes composed of three components (i.e., cholesterol, sorbitan monostearate, and dicetyl phosphate) showed degradation. This was attributed to the ionization of phosphoric acid moiety in the structure of surfactant (i.e., dicetyl phosphate) which led to the nucleophilic cleavage of GHK-Cu.[7] Given the advantages of delivery systems especially nanoparticles, much further attention should be paid to using such carriers for enhanced skin delivery of GHK and its derivative.

Pal-KTTKS is one of the first peptides introduced to the cosmetic market and most research on anti-wrinkle-peptides is about this palmitoylated peptide. Despite the attention paid to Pal-KTTKS and available information about this peptide, other anti-wrinkle peptides seem to have been somewhat neglected. We might need to turn our attention to other anti-wrinkle peptides including GHK and its derivatives as well.

GHK physicochemical properties and formulation challenges

The main purpose of preformulation studies is to optimize the process of developing an effective candidate molecule to a product. Unfortunately, there is a surprising lack of much information when it comes to preformulation studies on anti-wrinkle peptides including GHK derivatives. To the best of our knowledge, there is only one published study on the preformulation of metal complex derivative of GHK i.e., GHK-Cu[7] and almost no published preformulation study is available about the palmitoylated derivative of GHK.

In an attempt to conduct a preformulation study on GHK-Cu, the physicochemical properties of GHK-Cu, including the equilibrium aqueous solubility as well as the n-octanol/buffer distribution coefficients (logD) have been determined at ambient temperature.[7] Besides, the stability of GHK-Cu aqueous solution under various stress conditions such as 0.5 M hydrochloric acid and 0.5

M sodium hydroxide both at 60 °C, and 0.5% hydrogen peroxide at 22 °C were investigated. The compatibility of GHK-Cu with some common excipients used in preparation of noisome (i.e., Span 60, cholesterol and dicetyl phosphate) were also evaluated. The logD values for GHK-Cu at pH values of 7.4, 5.5, and 4.5 were -2.49, -2.38, and -2.49, respectively and the aqueous solubility of GHK-Cu was about 325 mg/mL. As expected, the results obtained regarding logD values along with the obtained aqueous solubility confirm that GHK-Cu is a hydrophilic peptide. In the case of forced degradation study, the worst stressor to induce hydrolytic cleavage was oxidative environment so that only 84.2% of GHK-Cu remained after one hour at 22 °C. The acidic stressor had the lowest impact on the degradation of GHK-Cu. The results of stability study showed that no detectable degradation at 60 °C over 14 days about aqueous solutions with pH 5.5 and 7.4. According to the authors, incorporation of GHK-Cu in a delivery system like noisome would be beneficial to protect as well as deliver a peptide with such physicochemical properties. Niosomes formulated using Span 60 and cholesterol without diacetyl phosphate, when stored at 40 °C, did not show any degradation for four weeks. However, using the surface charge modifying surfactant (i.e., diacetyl phosphate) along with Span 60 and cholesterol in the formulation reduced the stability. The lower stability of this formulation was attributed to the presence of phosphoric acid moiety in the structure of dicetyl phosphate which can promote the nucleophilic cleavage of the peptide in the basic solutions.[7] Although this study contains important and useful data, there are still some properties that should be experimentally determined for GHK-Cu including the physical form, pKa, hygroscopicity, particle size and shape, and so forth.

The common technique for identification of peptides is liquid chromatography–mass spectrometry[46] and anti-wrinkle peptides are not an exception. In addition to identification, the quantitative analysis of peptides is of great importance specially to assay their amount in formulations during stability studies. Besides, the achievement to a quantitative analysis method for measuring concentration of molecules permeated through skin is inevitable in permeation studies of anti-wrinkle peptides. It is worth noting that GHK-Cu has a quantitatively challenging assessment, due to the possibility of dynamic ligand exchange during permeation across the skin. Therefore, in some studies on GHK-Cu, the permeation of copper and GHK has been separately analyzed using atomic absorption spectroscopy and inductively coupled plasma mass spectrometry (ICP-MS) for Cu measurement and HPLC-UV for GHK measurement.[21,24]

Since many commercial cosmetic products of GHK derivatives are available on the market, such preformulation information would be very helpful for formulators not only for the promotion of current conventional formulations (e.g., serums, lotions, creams and so on) but also for the design of advanced formulations containing different types of delivery systems. The lack of literature in this field should be taken into consideration by researchers. As a guide and also to wrap this section, it is worth referring to two studies performed on preformulation of other peptides by our group. Mortazavi et al performed a preformulation study on KTTKS and Pal-KTTKS and studied their ultra violet absorption, structure, morphology, birefringence, partitioning, solubility, thermal behavior, surface activity, critical aggregation concentration, and stability.[47] Tabatabaie et al also synthesized different fatty acid conjugates of dipeptide KT and studied the mentioned physicochemical properties in the case of these conjugates.[35]

Concluding remarks

There is a growing interest and attention in using peptides as anti-wrinkle ingredients. GHK is a small bioactive peptide sequence capable of reducing wrinkles. From a physicochemical point of view, GHK does not possess desirable properties to show considerable skin permeability. However, its skin permeability could be promoted using copper complexation (GHK-Cu) and palmitoylation (Pal-GHK). Among many approaches of skin enhancement, only cell penetrating peptides and microneedles have been investigated to improve skin permeability of GHK derivatives so far. Other skin permeation enhancement strategies such as chemical permeation enhancers, physical enhancement methods (e.g., iontophoresis, sonophoresis, and electroporation) and encapsulation in nanocarriers, despite all their possible potentials, have not hitherto been investigated to increase permeability of these peptides.

Although preformulation studies are necessary to determine the physicochemical properties that may affect the formulations and their efficacy, only little is known about physicochemical properties of GHK-Cu and almost nothing about Pal-GHK. Therefore, the experimental physicochemical properties of Pal-GHK, as a widely used ingredient, must be explored. Also, no studies have been published on the possible side effects of these peptides. Given the importance of efficacy, permeability and safety information on anti-wrinkle peptide ingredients, further research on GHK derivatives is highly encouraged.

Acknowledgments

The authors kindly thank Mrs. L. Norouzi for her help in typing and administrative affairs

Authors' Contribution

Conceptualization: Seyedeh Maryam Mortazavi, Hamid Reza Moghimi.
Data curation: Seyedeh Maryam Mortazavi.
Formal analysis: Seyedeh Maryam Mortazavi.
Investigation: Seyedeh Maryam Mortazavi, Seyyed Ali Mohammadi Vadoud.
Methodology: Seyedeh Maryam Mortazavi, Hamid Reza Moghimi.

Review Highlights

What is the current knowledge?

√ Peptides are promising and attractive anti-wrinkle active ingredients.

√ Glycyl-histidyl-lysine peptide (GHK) is one of the most broadly promoted peptide for topical application.

√ GHK-Cu and Pal-GHK are metal complex and palmitoylated derivatives of GHK.

What is new here?

√ The necessity of considering the skin permeability as a prerequisite to GHK bioactivity.

√ The importance of permeation enhancement techniques for optimization of GHK and its derivatives delivery at the right concentration to the right place in the skin.

√ The formulation challenges of GHK and its derivatives.

√ Some suggestions for enhanced delivery of GHK.

Project administration: Seyedeh Maryam Mortazavi, Hamid Reza Moghimi.
Supervision: Hamid Reza Moghimi.
Writing-original draft: Seyedeh Maryam Mortazavi, Seyyed Ali Mohammadi Vadoud.
Writing-review and editing: Seyedeh Maryam Mortazavi, Hamid Reza Moghimi.

Competing Interests
Authors declare no conflict of interest.

Ethical Statement
None to be declared.

Funding
Not applicable.

References
1. Kennedy K, Cal R, Casey R, Lopez C, Adelfio A, Molloy B, et al. The anti-ageing effects of a natural peptide discovered by artificial intelligence. *Int J Cosmet Sci* **2020**; 42(4): 388-98. https://doi.org/10.1111/ics.12635
2. Skibska A, Perlikowska R. Signal Peptides - Promising Ingredients in Cosmetics. *Curr Protein Pept Sci* **2021**; 22(10): 716-28. https://doi.org/10.2174/1389203722666210812121129
3. Schagen SK. Topical Peptide Treatments with Effective Anti-Aging Results. *Cosmetics* **2017**; 4(2): 16. https://doi.org/10.3390/cosmetics4020016
4. Errante F, Ledwoń P, Latajka R, Rovero P, Papini AM. Cosmeceutical Peptides in the Framework of Sustainable Wellness Economy. *Front Chem* **2020**; 8: 572923. https://doi.org/10.3389/fchem.2020.572923
5. Mortazavi SM, Moghimi HR. Skin permeability, a dismissed necessity for anti-wrinkle peptide performance. *Int J Cosmet Sci* **2022**; 44(2): 232-48. https://doi.org/10.1111/ics.12770
6. Mehr A, Henneberg F, Chari A, Görlich D, Huyton T. The copper(II)-binding tripeptide GHK, a valuable crystallization and phasing tag for macromolecular crystallography. *Acta Crystallogr D Struct Biol* **2020**; 76: 1222-32. https://doi.org/10.1107/s2059798320013741
7. Badenhorst T, Svirskis D, Wu Z. Physicochemical characterization of native glycyl-L-histidyl-L-lysine tripeptide for wound healing and anti-aging: a preformulation study for dermal delivery. *Pharm Dev Technol* **2014**; 21: 1-9. https://doi.org/10.3109/10837450.2014.979944
8. Lane TF, Iruela-Arispe ML, Johnson RS, Sage EH. SPARC is a source of copper-binding peptides that stimulate angiogenesis. *J Cell Biol* **1994**; 125(4): 929-43. https://doi.org/10.1083/jcb.125.4.929
9. Rucker RB, Kosonen T, Clegg MS, Mitchell AE, Rucker BR, Uriu-Hare JY, et al. Copper, lysyl oxidase, and extracellular matrix protein cross-linking. *Am J Clin Nutr* **1998**; 67: 996s-1002s. https://doi.org/10.1093/ajcn/67.5.996S
10. Pickart L, Margolina A. Regenerative and Protective Actions of the GHK-Cu Peptide in the Light of the New Gene Data. *Int J Mol Sci* **2018**; 19(7): 1987. https://doi.org/10.3390/ijms19071987
11. Kechagia JZ, Ivaska J, Roca-Cusachs P. Integrins as biomechanical sensors of the microenvironment. *Nat Rev Mol Cell Biol* **2019**; 20(8): 457-73. https://doi.org/10.1038/s41580-019-0134-2
12. Kang Y-A, Choi HR, Na J-I, Huh CH, Kim M-J, Youn SW, et al. Copper-GHK increases integrin expression and p63 positivity by keratinocytes. *Arch Dermatol* **2009**; 301: 301-6. https://doi.org/10.1007/s00403-009-0942-x
13. Maquart FX, Bellon G, Chaqour B, Wegrowski J, Patt LM, Trachy RE, et al. In vivo stimulation of connective tissue accumulation by the tripeptide-copper complex glycyl-L-histidyl-L-lysine-Cu2+ in rat experimental wounds. *J Clin Invest* **1993**; 92(5): 2368-76. https://doi.org/10.1172/JCI116842
14. Abdulghani AA, Sherr A, Shirin S, Solodkina G, Tapia EM, Wolf B, et al. Effects of topical creams containing vitamin C, a copper-binding peptide cream and melatonin compared with tretinoin on the ultrastructure of normal skin - A pilot clinical, histologic, and ultrastructural study. *J Clin Outcomes Manag* **1998**; 4: 136-41.
15. Leyden J, Stephens T, Finkey M, Barkovic S. Skin Care Benefits of Copper Peptide Containing Eye Creams. . In: Proceedings of the American Academy of Dermatology 60th Annual Meeting; 22–27 February **2002**; New Orleans, LA, USA. p. 69.
16. Leyden J, Stephens T, Finkey M, Appa Y, Barkovic S. Skin Care Benefits of Copper Peptide Containing Facial Cream. In: Proceedings of the American Academy of Dermatology 60th Annual Meeting; 22–27 February **2002**; New Orleans, LA, USA. P. 6.
17. Guglielmi DA, Martinelli AM, Rissi NC, Cilli EM, Soares CP, Chiavacci LA. Synthesis of the peptide ac-wahx-kttks and evaluation of the ability to induce in vitro collagen synthesis. *Protein Pept Lett* **2016**; 23(6): 544-7. https://doi.org/10.2174/0929866523666160331144315
18. Jones RR, Castelletto V, Connon CJ, Hamley IW. Collagen Stimulating Effect of Peptide Amphiphile C16–KTTKS on Human Fibroblasts. *Mol Pharm* **2013**; 10(3): 1063-9. https://doi.org/10.1021/mp300549d
19. ACDLabs. ACD/ChemSketch. 12.01 ed. Toronto: Advanced Chemistry Development Inc; **2009**.
20. Dissette V, Bignozzi C, Manfredini S, Vertuani S, Valacchi G. Evaluation of the Transepidermal Penetration of a Carnosine Complex in Gel Formulation by 3D Skin Models. *Cosmetics* **2018**; 5: 67. https://doi.org/10.3390/cosmetics5040067
21. Hostynek JJ, Dreher F, Maibach HI. Human skin penetration of a copper tripeptide in vitro as a function of skin layer. *Inflamm Res* **2011**; 60(1): 79-86. https://doi.org/10.1007/s00011-010-0238-9
22. Park SI, An GM, Kim MG, Heo SH, Shin M. Enhancement of skin permeation of anti-wrinkle peptide GHKs using cell penetrating peptides. *Korean Chem Eng Res* **2020**; 58: 29-35. https://doi.org/10.9713/kcer.2020.58.1.29
23. Ferreira M, Magalhães C, Lobo J, Almeida I. Trending Anti-Aging Peptides. *Cosmetics* **2020**; 7: 91. https://doi.org/10.3390/cosmetics7040091
24. Li H, Low YS, Chong HP, Zin MT, Lee CY, Li B, et al. Microneedle-Mediated Delivery of Copper Peptide Through Skin. *Pharm Res* **2015**; 32(8): 2678-89. https://doi.org/10.1007/s11095-015-1652-z
25. Xie J, Bi Y, Zhang H, Dong S, Teng L, Lee RJ, et al. Cell-Penetrating Peptides in Diagnosis and Treatment of Human Diseases: From Preclinical Research to Clinical Application. *Front Pharmacol* **2020**; 11: 697. https://doi.org/10.3389/fphar.2020.00697
26. Derakhshankhah H, Jafari S. Cell penetrating peptides: A concise

review with emphasis on biomedical applications. *Biomed. Pharmacother* **2018**; 108: 1090-6. https://doi.org/10.1016/j.biopha.2018.09.097
27. Sadeghian I, Heidari R, Sadeghian S, Raee MJ, Negahdaripour M. Potential of cell-penetrating peptides (CPPs) in delivery of antiviral therapeutics and vaccines. *Eur J Pharm Sci* **2022**; 169: 106094. https://doi.org/10.1016/j.ejps.2021.106094
28. Gennari CG, Franzè S, Pellegrino S, Corsini E, Vistoli G, Montanari L, et al. Skin Penetrating Peptide as a Tool to Enhance the Permeation of Heparin through Human Epidermis. *Biomacromolecules* **2016**; 17(1): 46-55. https://doi.org/10.1021/acs.biomac.5b01524
29. Li QY, Zhang JN, Chen BZ, Wang QL, Guo XD. A solid polymer microneedle patch pretreatment enhances the permeation of drug molecules into the skin. *RSC Adv* **2017**; 7(25): 15408-15. https://doi.org/10.1039/C6RA26759A
30. Zhang S, Qiu Y, Gao Y. Enhanced delivery of hydrophilic peptides in vitro by transdermal microneedle pretreatment. *Acta Pharm Sin B* **2014**; 4(1): 100-4. https://doi.org/10.1016/j.apsb.2013.12.011
31. Li G, Badkar A, Nema S, Kolli CS, Banga AK. In vitro transdermal delivery of therapeutic antibodies using maltose microneedles. *Int J Pharm* **2009**; 368(1-2): 109-15. https://doi.org/10.1016/j.ijpharm.2008.10.008
32. Hao YY, Yang Y, Li QY, Zhang XP, Shen CB, Zhang C, et al. Effect of polymer microneedle pre-treatment on drug distributions in the skin in vivo. *J Drug Target*.**2020**; 28(7-8): 811-7. https://doi.org/10.1080/1061186X.2020.1757101
33. Mortazavi SM, Kobarfard F, Manafi A, Maibach H, Moghimi HR. Terpene conjugation: a novel approach for topical peptide delivery. *J Cosmet Sci* **2021**; 72(5): 547-56.
34. Mortazavi SM, Moghimi HR, Maibach HI. Chemical Modification: An Important and Feasible Method for Improving Peptide and Protein Dermal and Transdermal Delivery. In: Dragićević N, Maibach HI, editors. Percutaneous Absorption. Boca Raton: CRC Press; **2021**. p. 459-68. https://doi.org/10.1201/9780429202971
35. Sayed Tabatabaei M, Kobarfard F, Aboofazeli R, Ramezanpour S, Moghimi HR. Optimized cutaneous delivery of anti-wrinkle dipeptide kt via molecular modification: Preformulation, permeation, and the importance of conjugate chain length. *Bioimpacts*. **2025**; 15: 27558. https://doi.org/10.34172/bi.2023.27558
36. Lim SH, Sun Y, Thiruvallur Madanagopal T, Rosa V, Kang L. Enhanced Skin Permeation of Anti-wrinkle Peptides via Molecular Modification. *Sci Rep* **2018**; 8(1): 1596. https://doi.org/10.1038/s41598-017-18454-z
37. Goebel AS, Schmaus G, Neubert RH, Wohlrab J. Dermal peptide delivery using enhancer molecules and colloidal carrier systems--part I: carnosine. *Skin Pharmacol Physiol* **2012**; 25(6): 281-7. https://doi.org/10.1159/000341085
38. Gorouhi F, Maibach HI. Role of topical peptides in preventing or treating aged skin. *Int J Cosmet Sci* **2009**; 31(5): 327-45. https://doi.org/10.1111/j.1468-2494.2009.00490.x
39. Mohammed YH, Yamada M, Lin LL, Grice JE, Roberts MS, Raphael AP, et al. Microneedle enhanced delivery of cosmeceutically relevant peptides in human skin. *PloS One* **2014**; 9(7): e101956. https://doi.org/10.1371/journal.pone.0101956
40. Zhang S, Qiu Y, Gao Y. Enhanced delivery of hydrophilic peptides in vitro by transdermal microneedle pretreatment. *Acta Pharmaceutica Sinica B* **2014**; 4(1): 100-4. https://doi.org/10.1016/j.apsb.2013.12.011
41. Krishnan G, Roberts MS, Grice J, Anissimov YG, Moghimi HR, Benson HA. Iontophoretic skin permeation of peptides: an investigation into the influence of molecular properties, iontophoretic conditions and formulation parameters. *Drug Deliv Transl Res* **2014**; 4: 222-32. https://doi.org/10.1007/s13346-013-0181-8
42. Shokri J. Nanocosmetics: benefits and risks. *Bioimpacts* **2017**; 7: 207-8. https://doi.org/10.15171/bi.2017.24
43. Kim J-E, Oh G-H, Jang G-H, Kim Y-M, Park Y-J. Transformer-ethosomes with palmitoyl pentapeptide for improved transdermal delivery. *J Drug Deliv Sci Technol* **2019**; 52: 460-7. https://doi.org/10.1016/j.jddst.2019.04.039
44. Selvaraj K, Shin D, Yoo B. Effect of Partially Hydrolyzed Ginsenoside on In Vitro Skin Permeation and Retention of Collagen Pentapeptide (Palmitoyl-KTTKS). *Indian J Pharm Sci*. **2021**; 83: 76-83. https://doi.org/10.36468/pharmaceutical-sciences.752
45. Sommer E, Neubert RHH, Mentel M, Tuchscherer B, Mrestani Y, Wohlrab J. Dermal peptide delivery using enhancer molecules and colloidal carrier systems. Part III: Tetrapeptide GEKG. *Eur J Pharm Sci* **2018**; 124: 137-44. https://doi.org/10.1016/j.ejps.2018.08.034
46. Hollebrands B, Hageman JA, van de Sande JW, Albada B, Janssen H-G. Improved LC–MS identification of short homologous peptides using sequence-specific retention time predictors. *Analytical and Bioanalytical Chemistry* **2023**; 415: 2715-26. https://doi.org/10.1007/s00216-023-04670-2
47. Mortazavi SM, Kobarfard F, Maibach HI, Moghimi HR. Effect of palmitic acid conjugation on physicochemical properties of peptide kttks: A preformulation study. *J Cosmet Sci* **2019**; 70: 299-312.