REPORT

GHRP-6, a novel candidate for prevention and treatment of fibrotic disorders

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ABSTRACT

Fibrosis is defined as the pathological accumulation of extracellular matrix proteins (ECM) during the tissue repair response to an injury, which interferes with the functioning of the damaged organ or tissue. So far, there are no effective preventive or curative treatments. The growth hormone-releasing peptide 6 (GHRP-6) has anti-inflammatory, anti-oxidant and cytoprotective properties. Early signs of its possible anti-fibrotic effect were observed in a model of dilated cardiomyopathy in rats. This new property of the peptide was first studied in a model of liver cirrhosis in rats, in preventive and therapeutic scenarios. GHRP-6 reduced fibrotic induration in more than 75%, cords thickness and number of cirrhotic nodules by up to 60%, exerting besides a marked hepatoprotective effect. To assess its effect on the skin, GHRP-6 was applied in a simple wound model in rats, where it increased the rate of wound closure and decreased the inflammatory infiltrate. Subsequently, in a model of hypertrophic scarring in rabbits, the peptide prevented the appearance of keloids in more than 90% of the treated wounds. From the molecular point of view, GHRP-6 decreased the transcriptional expression of the pro-fibrotic genes TGFB1 and CTGF and induced the expression of the PPARG and MMP-13 genes, relevant for the inhibition of the pathological cumulative process. This work received the Annual Prize of the Cuban Academy of Sciences for the year 2016.

Keywords: fibrosis, hypertrophic scarring, keloid, GHRP-6, TGFB1, PPARG, wound healing

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RESUMEN

GHRP-6, un candidato novedoso para la prevención y tratamiento de desórdenes fibróticos. La fibrosis se define como la acumulación patológica de proteínas de la matriz extracelular (MEC) durante la respuesta reparativa tisular frente a una lesión, que interfiere con el funcionamiento del órgano o tejido dañado. Hasta el momento no existen tratamientos preventivos o curativos del todo eficaces. El péptido liberador de la hormona de crecimiento tipo 6 (GHRP-6) posee propiedades anti-inflamatorias, anti-oxidantes y citoprotectoras. Los primeros indicios de su posible efecto anti-fibrótico se observaron en un modelo en ratas de miocardiopatía dilatada. Esta nueva propiedad del péptido se estudió en un modelo de cirrosis hepática en ratas, en escenarios preventivo y terapéutico. El GHRP-6 redujo la induración fibrótica en más del 75%, el grosor de los cordones y el número de nódulos cirróticos hasta en un 60%, además de ejercer un marcado efecto hepatoprotector. Para evaluar su efecto en la piel, el GHRP-6 se aplicó en un modelo de herida simple en rata, donde incrementó la velocidad del cierre de las heridas y disminuyó el infiltrado inflamatorio. Posteriormente, en un modelo de cicatrización hipertrófica en conejos, el péptido previno la aparición de queloides en más del 90% de las heridas tratadas. Desde el punto de vista molecular, el GHRP-6 disminuyó la expresión transcripcional de los genes pro-fibróticos TGFB1 y CTGF e indujo la expresión de los genes PPARG y de MMP-13, relevantes para la inhibición del proceso acumulativo patológico. Este trabajo mereció el Premio Anual de la Academia de Ciencias de Cuba para el año 2016.

Palabras clave: fibrosis, cicatriz hipertrófica, queloide, GHRP-6, TGFB1, PPARG, cicatrización

*I*ntroduction

Fibrosis is defined as the pathological accumulation of extracellular matrix proteins (ECM) during tissue the repair response to mechanical, chemical or biological damage and interferes with the functioning of the affected organ or tissue [1]. Hepatic cirrhosis, cystic fibrosis and keloids are among the diseases displaying this type of process, which show a high incidence rate worldwide, particularly in developed countries, and with no efficacious preventive or curative treatments available [2].

A fortuitous finding made by our research group provided the first observations that the growth hormone-releasing peptide 6 (GHRP-6) establishes a cellular program of degradation or removal of excess ECM in parenchymal organs. GHRP-6 is a six-amino acids synthetic peptide with the sequence His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2, which was originally obtained as a synthetic derivative of intestinal metaencephalin [3]. At present, this peptide and some of its synthetic analogues are used as growth hormone secretagogues for the clinical diagnosis of different forms of dwarfism [4], and they have shown anti-inflammatory, anti-oxidant and cytoprotective properties with good safety profiles [5].

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In previous experiments, rats receiving repeated doses of doxorubicin for the induction of dilated cardiomyopathy, developed an intense interstitial fibrosis in the liver, kidneys and lungs. Subsequent histological examination showed a substantial decrease in the accumulation of collagen in animals treated with GHRP-6 versus those receiving a placebo solution (unpublished observations). To rigorously study these merely circumstantial findings, the anti-fibrotic effect of the peptide was characterized in several experimental models. First, the molecule was evaluated in a model of liver cirrhosis in rats mediated by carbon tetrachloride intoxication, in a preventive and two therapeutic scenarios. Subsequently, topical application of a viscous composition containing GHRP-6 was assessed in a simple wound model in rats and, afterwards, in a model of hypertrophic scarring in rabbit ears. The results obtained in these experiments will be briefly described below.

Results

Evaluation of the anti-fibrotic effect of GHRP-6 in a model of liver cirrhosis mediated by carbon tetrachloride intoxication in rats

Liver fibrosis was induced by the subcutaneous injection of CCl_4 twice a week for five months. The potential effect of the GHRP-6 intervention toward both fibrosis prevention and regression, was examined in two experimental blocks. The first one, developed during the five initial months, included the concomitant administration of GHRP-6 with CCl_4 to assess hepatic fibrosis prevention. In the second block of experiments, hepatic fibrosis was established during the first five months and then animals were divided into two groups of treatments: one received GHRP-6 alone for 15 days and the other received GHRP-6 and CCl_4 for 60 days. In these two schemes were carried out to assess the peptide potential in promoting cirrhosis regression.

The percentage of fibrosis, nodularity and septal thickness were quantified by histomorphometric studies and ascites or portal dilation by ultrasound. The biochemical profile and the parameters of oxidative stress in serum, as well as the genes and proteins expression pattern, were determined by reverse transcription associated to polymerase chain reaction and by immunohistochemistry.

Treatment with GHRP-6 significantly reduced fibrosis markers such as fibrosis index, portal diameter, presence of ascites, thickness of the walls and number of nodules per field, both from the ultrasonographic and histological point of view (Table 1 and Table 2, respectively), in both concomitant and therapeutic approaches. Representative images of the macroscopic and histological structure of livers before and after GHRP-6 peptide administration are shown in Figure 1.

From the functional point of view, it was observed that the peptide significantly attenuated the dramatic increase in the levels of the transaminases aspartate-aminotransferase and alanine-aminotransferase, which occurred in the animals receiving the hepatotoxic agent. On the other hand, GHRP-6 significantly reduced the levels of oxidative stress markers as compared to saline-treated animals ($p < 0.01$) in the three experimental schemes, including the levels of total hydroperoxides, advanced protein oxidation products, malondialdehyde and the lipid peroxidation potential. Concurrently, GHRP-6 increased the activity of the anti-oxidant enzymes catalase and su-

Table 1. Hepatic ultrasound results of GHRP-6-treated mice†

Experimental groups	N	UFI	Portal diameter (mm)	Ultrasound ascites (%)
Intact control	7	0	0.71 ± 0.05	0
CCl_4 + GHRP-6	12	2.50 ± 0.30	0.80 ± 0.06	2 (17)
CCl_4 + Saline	12	5.93 ± 0.79***	1.12 ± 0.14*	8 (67)
GHRP-6 60 d	12	4.80 ± 0.42	0.96 ± 0.08	3 (25)
Saline 60 d	12	7.00 ± 0.29***	1.34 ± 0.13*	8 (67)

† Ultrasounds to CCl_4 + GHRP6 and CCl_4 + Saline groups were conducted at the fifth experimental month while to GHRP6-60d and Saline-60d groups were at the seventh month. Portal diameter value reported for Intact control group was obtained at seventh month. UFI: Ultrasonic fibrosis index. Data from UFI and portal diameter are presented as average ± SEM by group. Ascites results are indicated as the total number of animals and as percentage, by group. Ultrasound ascites data include the clinical ascites. */*** indicate significant differences between the GHRP6-treated animals and their counterpart Saline groups for at least $p < 0.05$.

Table 2. Histomorphometric analyses of GHRP-6-treated mice

Experimental groups	N	Septae thickness	Nodules per mm^2	Fibrosis (%)
CCl_4 + GHRP-6	12	10.97 ± 1.66***	0.82 ± 0.23***	1.99 ± 0.31***
CCl_4 + GHRP-6	12	72.13 ± 7.85	5.58 ± 0.52	16.80 ± 0.63
GHRP-6-15 d	10	47.31 ± 4.62**	3.80 ± 0.41	9.80 ± 1.15***
Saline-15 d	10	71.63 ± 4.67	5.00 ± 0.44	15.63 ± 0.67
GHRP-60 d	12	34.62 ± 4.36***	2.24 ± 0.42***	4.93 ± 0.65***
Saline-60 d	12	102.73 ± 8.14	5.90 ± 0.43	19.37 ± 0.68

† The histomorphometric analyses were conducted using Mallory staining slides. Data are presented as average ± SEM by group. The septae thickness was determined in microns. **/*** indicate significant differences between GHRP6-treated animals and their respectively Saline groups for at least $p < 0.01$.

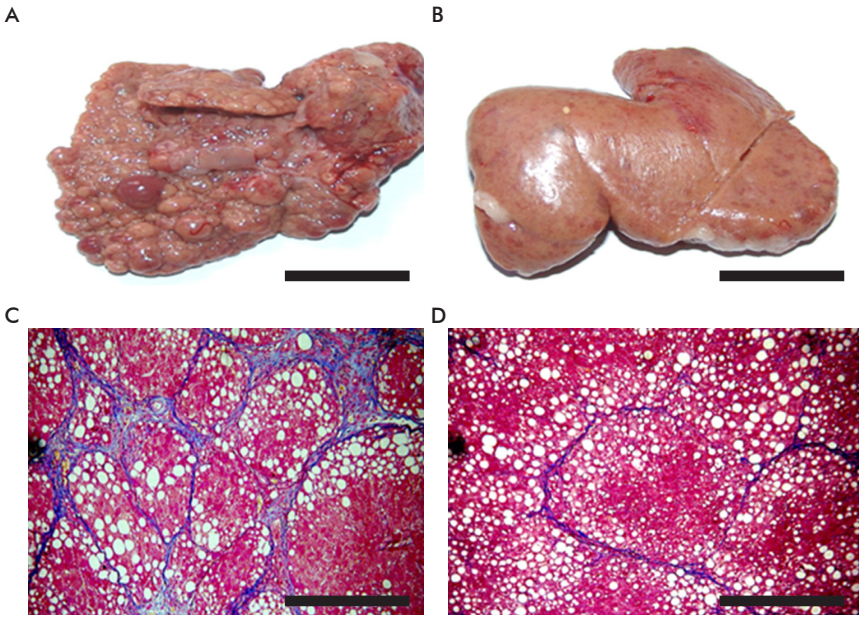

Figure 1. Images representative of livers from mice treated with GHRP-6 Saline, 60 days after treatment. A) Macroscopic image, saline administration. B) Macroscopic image, GHRP-6 treatment. C) Histologic image, saline administration. D) Histologic image, GHRP-6. Histological analyses were conducted by Mallory's trichrome stain, and images were obtained with 20×. The bar stands for 10 mm (A and B), and 200 μm (C and D).

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peroxide dismutase ($p < 0.05$). In general, we could conclude that the GHRP-6 intervention removed and controlled the pathological deposition of collagen and ECM in the hepatic parenchyma, while exerting marked hepatoprotective and proliferation promoter effects [7].

Topical application of a carboxymethylcellulose gel containing GHRP-6 in the simple open excision wound model in rats

More than a decade ago, CD36 was identified as one of the GHRP-6 receptors [8]. Serendipitous observations of our laboratory indicated that CD36 mRNA transcript appeared abundantly represented in clinical samples of granulation tissue of either acute (deep burn injuries) or chronic (pressure ulcers) wounds. This finding incited us to speculate about the possible effect of GHRP-6 topical administration on the wound healing process.

A formulation of GHRP-6 (400 µg/mL) in 1 % carboxymethylcellulose gel (CMC) was applied for 4 days in controlled full-thickness skin wounds, which were surgically made on the back of male Wistar rats. As shown in Figure 2, the administration of GHRP-6 significantly increased wound closure rate as compared to vehicle (CMC 1 %), starting 24 h after the initial administration of the peptide ($p = 0.016$) and until the end of the experiment ($p < 0.0001$). Wounds treated with GHRP-6 showed lower amounts of inflammatory infiltrate and attained a higher degree of organization of their ECM, due to a lower accumulation of fibrin and the presence of thinner and horizontally distributed collagen strands. No statistically significant differences were observed in the number of active blood vessels (Table 3).

At the molecular level, GHRP-6 reduced the transcriptional expression of genes encoding tumor necrosis factor converting enzyme (Adam17; $p = 0.0306$), transforming growth factor β1 (Tgfb1; $p = 0.0171$) and connective tissue growth factor (Ctgf; $p = 0.001$). These effects were translated into a decreased expression of genes coding for ECM proteins and for myofibroblast marker proteins [9].

Demonstration of the anti-fibrotic effect of GHRP-6 in the keloid model on the inner surface of the rabbit ear

White male New Zealand rabbits (4.3-4.5 kg) were used in four independent and extemporaneous experiments. Three to four wounds were created on the ventral side of each ear, down to the surface of the cartilage, using a 6 mm diameter punch biotome. Rabbits were randomly assigned to either GHRP-6 (400 µg/mL) treatment or 1 % CMC placebo gel. Treatments were initiated immediately after surgery and continued thereafter until day 30 and the animals remained in observation for another 20 days after GHRP-6 administration had been completed. The most notable effect of the GHRP-6 intervention was the prevention of hypertrophic scar formation. As shown in Table 4, treatment with the peptide abrogated the debut of keloids in 90.5 % of the treated wounds. Conversely, 87.5 % of the wounds that received the viscous solution of 1 % CMC without

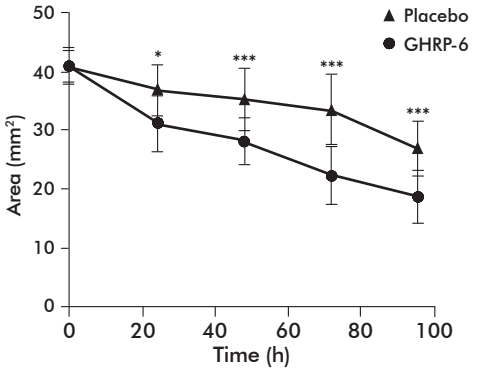

Figure 2. GHRP-6 accelerated wound closure. Differences in wounded area reduction appeared since the first 24 hours of postinjury. GHRP-6-induced contraction remained stable until hour 96, when the animals were terminated. Two-way ANOVA (*$p = 0.016$, ***$p < 0.001$). Reproduced from: Mendoza Marí Y, *et al*. Plast Surg Int. 2016;2016:4361702.

Table 3. Impact of GHRP-6 topical administration on inflammation and fibroangiogenesis†

Group	Inflammatory cells	Active vessels	Dermal matrix reconstitution
GHRP-6	7.86 ± 2.41***	8.34 ± 3.02	1.90 ± 0.36***
Placebo	15.74 ± 3.91	8.38 ± 2.89	2.49 ± 0.38

† Reproduced from: Mendoza Marí Y, *et al*. Plast Surg Int. 2016;2016:4361702.
***$p = 0.001$. Two-tailed unpaired Student's t-test.

Table 4. Impact of GHRP-6 topical administration on inflammation and fibroangiogenesis†

Treatment	Number of wounds	Hypertrophic phenotype	Normal phenotype	Scar elevation index
GHRP-6	84	8 (9.5%)	76 (90.5%)	1.12 ± 0.11***
Placebo	80	70 (87.5%)	10 (12.5%)	1.67 ± 0.15

† Scar elevation index was measured in 8 nonresponsive wounds of the GHRP-6 treated group. Reproduced from: Mendoza Marí Y, *et al*. Plast Surg Int. 2016;2016:4361702.
***$p = 0.001$. Two-tailed unpaired Student's t-test.

the peptide evolved into a hypertrophic scar, with a nipple-like appearance, reddened and firm in touch (Figure 3).

GHRP-6 appears to primarily reduce the local hypercellularity associated with cartilage perichondrium cells and the resulting accumulation of ECM (Figure 4). Consequently, the scar elevation index was

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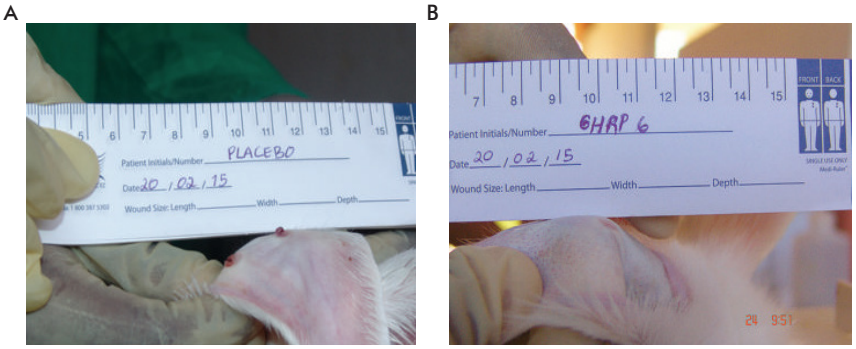

Figure 3. Topical GHRP-6 improved the macroscopic aspect of the wounds. A) Representative wounds that evolved to hypertrophic scars (HTS). B) Representative image of the effect of GHRP-6 administration. Reproduced from: Mendoza Marí Y, *et al*. Plast Surg Int. 2016;2016:4361702.

significantly lower ($p = 0.001$) in treated wounds (1.12 ± 0.11) than those receiving the vehicle (1.62 ± 0.15). At the molecular level, GHRP-6 significantly reduced the transcriptional expression of Tgfb1 and Ctgf ($p < 0.05$) and increased the expression of the PPARγ transcriptional factor ($p = 0.016$) [9].

Relevance of the study

The results presented above constitute the first evidence of a new pharmacological property for GHRP-6: its ability to inhibit synthesis and excessive accumulation of extracellular matrix proteins. This effect was demonstrated in parenchymal (liver) and peripheral (skin) organs and in prophylactic and therapeutic scenarios. On the other hand, a new mechanism of action for GHRP-6, based on the induction of PPARγ, is described. Evidence obtained indicates that GHRP-6 is potentially useful for the prevention/treatment of liver fibrosis, keloids and hypertrophic scars and broadens the spectrum of therapeutic possibilities to other fibrotic and accumulation diseases.

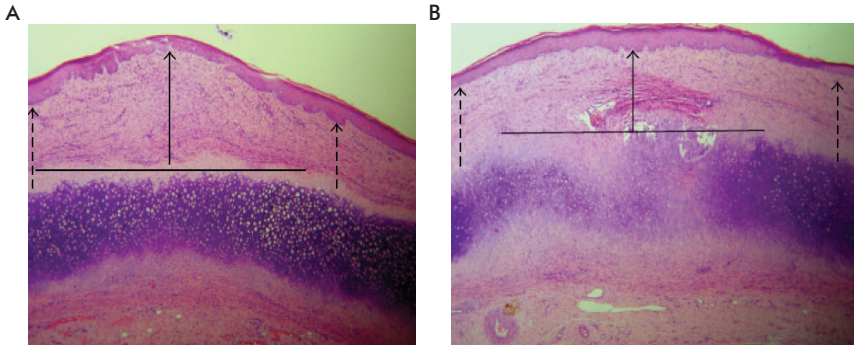

Figure 4. Microscopic aspect of the rabbits' ears wounds. A) Representative image of "nipple" like lessions in rabbits receiving placebo. B) Representative image of the effect of GHRP-6 administration. Images magnification: 20x. Reproduced from: Mendoza Marí Y, *et al*. Plast Surg Int. 2016;2016:4361702.

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