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Islet neogenesis-associated protein (INGAP): The role of its endogenous production as a positive modulator of insulin secretion

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ABSTRACT

Islet neogenesis-associated protein (INGAP) is a peptide found in pancreatic exocrine-, duct- and islet- non-β-cells from normal hamsters. Its increase induced by either its exogenous administration or by the overexpression of its gene enhances β-cell secretory function and increases β-cell mass by a combination of stimulation of cell replication and islet neogenesis and reduction of β-cell apoptosis. We studied the potential modulatory role of endogenous INGAP in insulin secretion using two different experimental approaches. Hamster islets transfected with INGAP-small interfering RNA (INGAP-siRNA) were used to study glucose-stimulated insulin secretion (GSIS). In parallel, freshly isolated islets were incubated with high glucose and the same concentration of either a specific anti-INGAP rabbit serum or normal rabbit serum. INGAP-siRNA transfected islets reduced their INGAP mRNA and protein content by 35.1% and 47.2%, respectively whereas GSIS decreased by 25.8%. GSIS by transfected islets attained levels comparable to those recorded in control islets when INGAP pentadecapeptide (INGAP-PP) was added to the culture medium. INGAP antibody in the medium decreased significantly GSIS in a dose-dependent manner. These results indicate that endogenous INGAP plays a "physiological" positive modulatory role in insulin secretion, supporting its possible use in the treatment of prediabetes and Type 2 diabetes.

1. Introduction

Islet neogenesis-associated protein (INGAP) was initially identified as the active component of a protein complex called ilotropin found in cellophane-wrapped pancreas heads of normal hamsters [1]. INGAP gene was thereafter cloned [2] and its mRNA was identified in pancreatic islets and exocrine cells [3]. INGAP-immunoreactive positive cells were further identified in pancreatic exocrine-, duct- and islet- non-β-cells from normal hamsters and normal adult rats [4]. A pentadecapeptide having the 104–118 amino acid sequence of INGAP (INGAP-PP) reproduced the stimulatory effect of the intact molecule upon thymidine incorporation into duct cells and a duct cell line [2].

The addition of INGAP-PP to cultured islets isolated from either neonatal or normal adult rats induced the expression of several genes related to β-cell function [5–10] and increased significantly β-cell size and insulin release in response to glucose and amino acids [11]. On the other hand, INGAP transgenic mice presented an improved glucose tolerance test and significantly delayed the appearance of diabetes in streptozotocin (STZ)-treated mice [12,13].

Islets isolated from normal hamsters treated with INGAP-PP for 10 days increased significantly glucose-induced insulin release; they also showed an increased β-cell replication rate and a decreased β-cell apoptosis, leading to a significant increase in their mass [9]. These changes can be partially ascribed to the INGAP-PP effect upon key gene expression: it increases the mRNA concentration of Ngn-3 and Pdx-1 [9,14] and decreases that of p38MAPK and JNK [15]. On the other hand, INGAP-PP administration to mice with STZ-induced diabetes also increased β-cell mass and reduced the serum glucose level in 50% of the animals [16]. Further, INGAP-PP administration in a clinical trial increased C-peptide secretion in people with Type 1 diabetes and improved glycemic control in people with Type 2 diabetes [17]. The biological effects of INGAP and its potential effectiveness in the treatment of human diabetes have been recently reviewed [18].

Based on the data so far recorded, the effect of INGAP-PP upon β-cell mass and function appears to be a pharmacological rather than a physiological one; thus, we tested the possible modulatory role of endogenous INGAP in insulin secretion. For this purpose, we studied the effect of INGAP upon glucose-induced insulin secretion (GSIS) by using two different experimental approaches: a) islets from normal hamsters were transfected with INGAP-small interfering RNA (siRNA), and b) freshly

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isolated islets were challenged with glucose in the presence of a specific INGAP-antibody. Both conditions significantly decreased the release of insulin in response to glucose.

2. Materials and methods

2.1. Chemicals and drugs

Collagenase was from Serva Feinbiochemica (Heidelberg, Germany); bovine serum albumin (BSA) fraction V, anti β-actin antibody and other reagents were from Sigma Chemical Co. (St. Louis, MO, USA). The INGAP antiserum was a gift from Dr. A. Vinik (Strelitz Diabetes Institutes, The Research Institute at Eastern Virginia Medical School Norfolk, USA). Chemically synthesized, double-stranded siRNA targeting hamster INGAP (INGAP-siRNA) and the control non-targeting siRNA fused to the green fluorescent protein (GFP, Block-iT Fluorescent Oligo) were purchased from Invitrogen (San Diego, CA, USA). The sequence of INGAP-siRNA was the following: sense, 5′ggaaguggagcaguuccaau gugcu3′ and antisense, 5′agcacauuggaacugcuccacuucc3′ (U41738.1, AY184211.1). The AccuTarget biotin-labeled negative control siRNA (5′-CCUACGCCACCAAUUUCGUdTdT-3′) (Bioneer Inc, Korea) that exhibits no homology to any genome sequence was used as a non-silencing reference. INGAP-PP (Ile-Gly-Leu-His-Asp-Pro-Ser-His-Gly-Thr-Leu-Pro-Asn-Gly-Ser) was kindly provided by Dr. G. Alexander Fleming (Kinexum LLC, Harper's Ferry, West Virginia, USA).

2.2. Animals

All experiments were performed with adult male Syrian hamsters (90–110 g bw) maintained under controlled conditions at 23 °C and a 12-h light–dark cycle (0600 h–1800 h), with free access to standard commercial food and tap water. Animal experiments were performed according to the "Ethical Principles and Guidelines for Experimental Animals" (3rd ed., 2005) from the Swiss Academy of Medical Sciences (mail@samw.ch).

2.3. Islet isolation, culture and transfection

Islets from normal hamsters were isolated by collagenase digestion [19] and cultured for 3 days in RPMI-1640 medium with 11 mM glucose, 5% fetal bovine serum, 100 IU penicillin/ml and 100 mg streptomycin/ml at 37 °C.

After this period, the culture medium was replaced by antibiotic-free media. All the islets were treated with Lipofectamine 2000 (Invitrogen) diluted in Opti-MEM reduced Serum Medium (Invitrogen), plus 0.1 μM INGAP-siRNA (INGAP-siRNA group), 0.1 μM AccuTarget biotin-labeled non-silencing control siRNA (*NSC group*), and without any siRNA (*control group, C*). Both control groups were included to demonstrate that the results obtained in INGAP-siRNA transfected islets were not due to the toxicity of the transfection process.

After 4 h under those conditions, islets from each group were further divided into two sub-groups and cultured overnight at 37 °C in the presence or absence of 10 μM INGAP-PP in complete RPMI medium.

Transfection efficiency was tested by fluorescence microscopy in islets transfected with 0.1 μM Block-iT siRNA-GFP (a non targeting siRNA carrying the green fluorescent protein from Invitrogen) in Lipofectamine 2000 diluted in Opti-MEM reduced Serum Medium.

2.4. Quantitative real-time PCR

Total RNA was isolated from NSC, C and INGAP-siRNA islets in 5 independent experiments using Trizol reagent (Invitrogen), following the manufacturer's instructions. RNA integrity was checked by agarose–formaldehyde gel electrophoresis. Possible contamination with protein or phenol was controlled by measuring the 260/280 nm absorbance ratio [20], while DNA contamination was avoided treating the sample with DNase I (Invitrogen); 1 μg of total RNA was used for reverse transcription with SuperScript III Reverse Transcriptase (Invitrogen) and oligo-dT. Specific pairs of primers based on hamster cDNA sequences were designed as follows: INGAP (U41738.1) forward primer ′aacctgtcctcaaggctctg3′; reverse primer 5′tcagcacattggaactg ctc3′. To avoid genomic DNA contaminations, INGAP primers were designed from different exons on the basis of the gene sequence published by Taylor-Fishwick et al. [21]; thus, forward is from exon 3 and reverse is from exon 5.

Real-time PCRs were run in triplicate using 1/40th of the cDNA per reaction and 1 μmol/l forward and reverse primers with PlatinumSYBR Green qPCR Supermix-UDG (Invitrogen) in the iCycler 5 (BioRad). The cycling profile used was: 1 cycle of 2 min at 50 °C (uracil-DNA glycosylase (UDG) activation), 1 cycle of 2 min at 95 °C (DNA denaturation and UDG inactivation), 40 cycles of 15 s at 95 °C and 30 s at 60 °C, 1 cycle of 1 min at 95 °C followed by a melting curve from 55 °C to 90 °C. Quantified values for each gene were normalized against the housekeeping gene β-actin (VO1217; forward primer, 5′agagggaaattgtgcgtgac3′; reverse primer, 5′ttgccaatggtgatgacctg3′) using the individual efficiency calculated with a standard curve for each gene. The standard curve was done using a mixture of islet cDNA samples obtained from several normal hamsters.

2.5. Western blot

Cultured islets from all experimental groups (NSC, C and INGAP-siRNA) were homogenized in 100 mM Tris, pH 7.4, 10 mM EDTA, 1% Triton-X100, 2 mM phenyl-methylsulphonil-fluoride, 0.1 mg/ml aprotinin, 100 mM sodium pyrophosphate, 10 mM sodium vanadate and 100 mM sodium fluoride. Proteins from islet homogenates (30 μg) were fractioned under reducing conditions by SDS/PAGE (12% gel) and electroblotted to polyvinylidene difluoride transfer membranes (Amersham Hybond™-P, GE Healthcare, UK). The protein loaded onto the gel was quantified using the Bio-Rad protein assay [22]. Nonspecific binding sites were blocked with TBS buffer containing 3% *w/v* BSA at 4 °C for 90 min for INGAP quantification or with a non-fat milk solution at 4 °C overnight for β-actin quantification (housekeeping protein). The membranes were then incubated overnight with INGAP antiserum (1:1000 dilution) or with β-actin antibody (1:10,000 dilution) at 4 °C for 90 min. After rinsing with T-TBS, the blots were incubated with peroxidase-conjugated second antibody (goat anti-rabbit IgG-HRP sc-2004; Santa Cruz Biotechnology, CA, USA) for 90 min at room temperature. For β-actin, the horseradish-peroxidase-conjugated anti-mouse IgG antibody (sc-2005; Santa Cruz Biotechnology, CA, USA) was used as secondary antibody. INGAP protein level was measured by using an enhanced chemiluminescence detection system (ECL Prime, Amersham, GE Healthcare, UK) and C-Digit Blot Scanner (Li-Cor Biosciences). Finally, band density was quantified using Image Studio Digits 3.1 software.

2.6. Insulin secretion

Islets from each experimental group (NSC, C and INGAP-siRNA) were cultured overnight in the absence or presence of 10 μg/ml INGAP-PP and then incubated for 60 min at 37 °C in KRB, pH 7.4, previously gassed with a mixture of CO_2/O_2 (5/95%), containing 1.0% (*w/v*) BSA and 3.3 or 16.7 mM glucose.

In another set of experiments, freshly isolated islets from normal hamsters were incubated in the media described above but in the presence of four different dilutions of a specific rabbit INGAP anti-serum (1:2000; 1:1000; 1:500 and 1:100), in the presence or absence of 10 μg/ml INGAP-PP. To test the specificity of the antibody used, normal islets were simultaneously incubated in the presence of normal rabbit serum diluted 1:100 (the lowest dilution tested of the specific anti-INGAP serum).

In both cases, aliquots from the medium were taken after the incubation and kept frozen for insulin measurement by radioimmunoassay [23], using an antibody that specifically recognizes hamster insulin.

Although the islets used for all the assays had a similar shape and size, results were expressed as pg of insulin released per ng of islet DNA in order to avoid differences ascribed to the possible uneven cell content of the islets from the different experimental groups. Islet DNA content was measured by a fluorometric assay [24] in islet homogenates obtained from all experimental groups.

2.7. Statistical analysis

Quantitative data were expressed as means ± SEM. Statistical significance ($P < 0.05$) was determined using the ANOVA for independent samples followed by Bonferroni test and the Student's *t*-test.

3. Results

3.1. Transfection efficiency and INGAP-siRNA effectiveness

Transfection of isolated islets with the non-targeting siRNA fused to GFP showed that they have taken up the material transfected, at least in those cells located in the islet periphery (Fig. 1A).

INGAP mRNA concentration was significantly reduced in INGAP-siRNA transfected islets ($P < 0.05$): 35.1% and 37.1% with respect to that recorded in non-silencing siRNA transfected islets (NSC) and in non transfected islets (C), respectively (Fig. 1B). INGAP protein concentration however underwent a larger reduction, 47.2% and 55.2% lower than that recorded in NSC and C islets, respectively; Fig. 1C).

3.2. Effect of INGAP-siRNA upon insulin secretion

Islets transfected with INGAP-siRNA did not show any change in insulin secretion elicited by 3.3 mM glucose concentration (Fig. 2), but they released significantly less insulin when challenged with 16.7 mM glucose (25.8% and 19.5% less than NSC and C islets, respectively; $P < 0.05$; Fig. 3).

The overnight addition of INGAP-PP to the culture medium of islets transfected with the specific siRNA significantly increased insulin secretion ($P < 0.05$) in response to 16.7 mM glucose (Fig. 2). Such addition did not affect insulin secretion in response to 3.3 mM glucose (data not shown).

3.3. Insulin secretion in the presence of INGAP antiserum

Islets isolated from normal hamsters incubated in the presence of an specific INGAP-antiserum (1:2000; 1:1000; 1:500 and 1:100 final dilution) released a significantly lower amount of insulin ($P < 0.05$) in response to 16.7 mM glucose in a dose-dependent manner (20.9%; 30.8% and 36.7% for 1:1,000; 1:500 and 1:100 respectively; Fig. 3A). This lowering effect was not observed when the islets were incubated either in the presence of the highest INGAP-antibody dilution tested (1:2000) or of 1:100 normal rabbit serum. As shown in Fig. 3B, the simultaneous presence of INGAP-PP and of the anti-INGAP serum in the

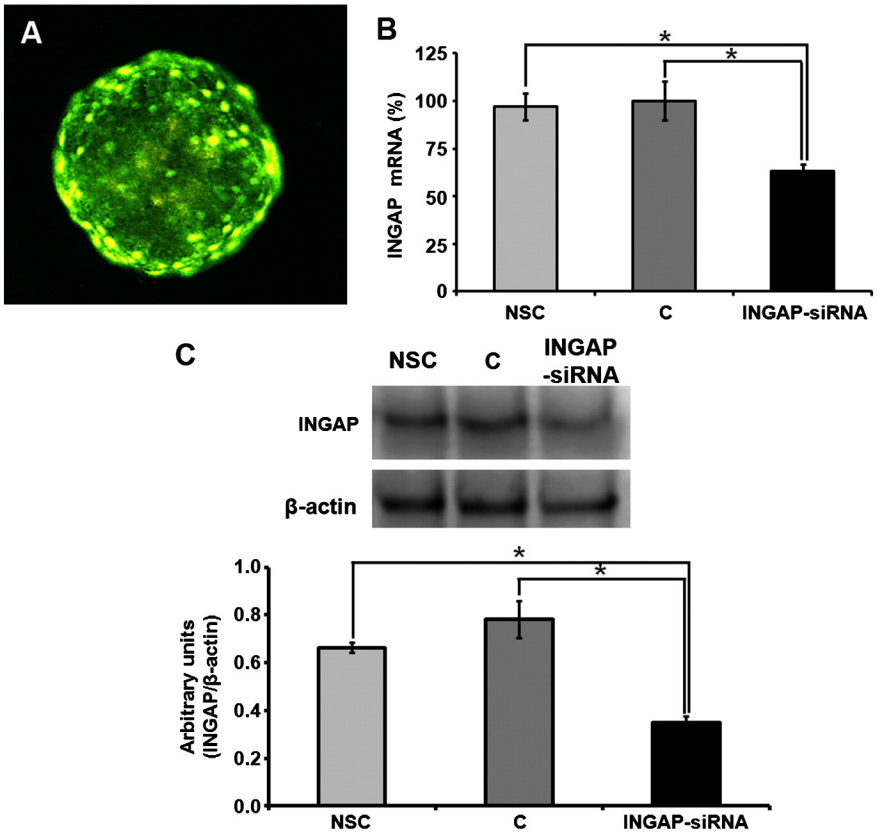

Fig. 1. (for color reproduction available on the Web). Effectiveness of transfection. (A) Whole islet transfected with non-targeting GFP-siRNA showing positively stained cells in the islet periphery. × 40. (B) INGAP mRNA relative expression (real time PCR) in islets transfected with a non-silencing control-siRNA (NSC, white bar), treated with all the reagents used in the transfection protocol but without siRNA (C, gray bar) and transfected with INGAP-siRNA (black bar). Bars represent mean values ± SEM of five independent experiments (each experiment by triplicate). *$P < 0.05$. (C) INGAP protein level measured by Western blot in islet homogenates from NSC (white bar), C (gray bar) and INGAP-siRNA (black bar). β-actin was used as housekeeping protein. A representative blot from three independent experiments is shown. Bars below the blot represent mean values ± SEM expressed in arbitrary units as the ratio between INGAP and β -actin band intensity. $P < 0.05$, §vs. NSC and *vs. C.

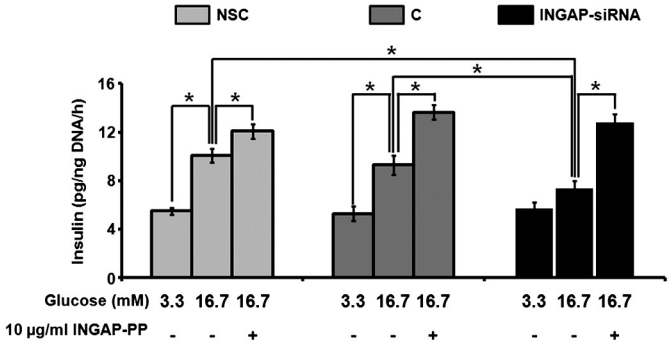

Fig. 2. Effect of INGAP mRNA blockage upon in vitro GSIS. Insulin secretion in response to 3.3 and 16.7 mmol/l glucose by islets cultured overnight in the absence or presence of 10 µg/ml INGAP-PP. NSC: islets transfected with a non-silencing control-siRNA (white bar), C: islets treated with all the reagents used in the transfection protocol but without siRNA (gray bar), and INGAP-siRNA: islets transfected with INGAP-siRNA (black bar). Bars represent mean values ± SEM from five independent experiments (8 tubes per condition per experiment, $n = 40$ in each case). $P < 0.05$, for all experimental groups: ^vs. 3.3 mM glucose and #vs. 16.7 mM without INGAP-PP; between experimental groups: §vs. NSC and *vs. C.

incubation media, blunted the inhibitory effect of the antibody (diluted 1:500 and 1:100) upon insulin secretion.

4. Discussion

We currently transfected intact islets isolated from normal hamsters with a specific siRNA targeted against INGAP in order to test its potential physiological effect upon β-cell secretory function. Effective transfection was demonstrated in the periphery of the islets by visualization of the green fluorescence present in islets transfected with the non-targeting siRNA fused to GFP; this topographic islet area corresponds to the position of the majority of the INGAP-positive cells, as previously reported [3,25,26]. Consequently, any functional change observed after INGAP-siRNA transfection might be reasonably considered as a specific effect rather than as an artifact. Supporting this assumption, INGAP specific siRNA transfection induced a simultaneous partial and significant reduction in both INGAP mRNA (35.1%) and protein (47.2%) concentration as well as in glucose-induced insulin secretion (25.8%). This partial reduction effect might be ascribed to the incomplete penetrance of the transfected material, though INGAP expression has been largely reported as limited to the peripheral islet cells [3,25,26]. We cannot discard however, that lower INGAP levels expressed in inner islet cells (not detected by immunostainig) [3] could also contribute to the partial inhibition recorded, since those cells are not properly reached by transfection,

GSIS recorded in INGAP-siRNA-transfected islets was significantly lower than that recorded in both control groups (NSC and C). In fact, when glucose concentration in the incubation medium rose from 3.3 to 16.7 mM, both control groups increased significantly the release of insulin (83% and 77%, respectively), whereas only a modest 29% increase was obtained in INGAP-siRNA transfected islets. The fact that the overnight addition of INGAP-PP to the culture medium of transfected islets was sufficient to increase GSIS to levels comparable to those recorded in control islets lends further support to this concept. Thus, the negative functional impact of INGAP-siRNA could be ascribed to the lower production/availability of endogenous INGAP to exert its enhancing effect upon the different mechanisms involved in the process of insulin secretion [6–9,11]. In agreement with our results, it has been reported that a siRNA targeting INGAP can effectively down-regulate INGAP expression and inhibit the proliferation of INS-1 cells [27].

A similar decrease in insulin response to the high glucose stimulus was observed when freshly isolated islets were incubated in the presence of a specific INGAP-antiserum in the medium. In this case, the effect of the endogenous INGAP was blocked by its specific antibody and was therefore no longer able to interact with its islet cell receptors [28]. This inhibitory effect triggered by anti-INGAP serum was specific since a) it depends on concentration of anti-INGAP serum but it is not affected by the presence of normal rabbit serum and b) it was prevented by the presence of INGAP-PP in the incubation medium.

As mentioned above, INGAP is mostly expressed in islet non-β-cells; therefore, we may assume that its effect upon insulin secretion depends on some type of paracrine regulatory mechanism. The results obtained with the INGAP antiserum and published literature supports this assumption, namely, a) the presence of specific low-affinity INGAP receptors in the islets [28], b) the paracrine effect of INGAP upon islet cell proliferation [29], and c) the fact that the potent insulin-releasing action of glucose in intact islets not only depends on glucose metabolism in pancreatic β-cells, but also on the modulatory paracrine regulation exerted by other hormones produced by non-β-cells such as glucagon through the modification of cAMP levels [30]. Consequently, the current data strongly suggest the existence of a physiological and paracrine tonic effect of INGAP upon insulin secretion. Whether INGAP exerts a similar tonic effect upon glucagon secretion remains an open question that merits further studies.

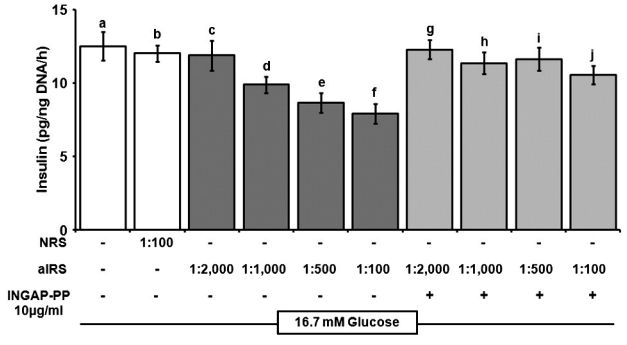

Fig. 3. Effect of INGAP-specific antiserum upon in vitro GSIS. Islets isolated from normal hamsters were incubated for 60 min in the presence of 16.7 mM glucose alone (first column) or with either 1:100 normal rabbit serum (NRS; second column) or specific anti-INGAP rabbit serum (aIRS; 1:2000; 1:1000; 1:500 and 1:100) in the absence or presence of 10 µg/ml INGAP-PP. Insulin released was determined by RIA and expressed as pg per ng of islet DNA in 1 h to avoid differences due to the possible uneven cell number of the islets used. Bars represent mean values ± SEM of three independent experiments (8 tubes per condition per experiment, $n = 24$ in each case). a vs. d, e, f; b vs. d, e, f; c vs. e, f; d vs. f; e vs. i; f vs. j; in all cases $P < 0.05$.

5. Conclusions

Evidence of the effects of INGAP upon islet function and mass was obtained using different experimental approaches, namely, addition of either INGAP-PP or the whole protein to the culture/incubation medium of islets, its administration to normal or diabetic animals, and targeting INGAP expression in transgenic mice [2,6,11–13,16,25]. However, no previous evidence supports the possible physiological role of the INGAP protein in normal β-cell secretory function. Our current results – obtained using two different experimental approaches not previously tested – strongly suggest that INGAP plays such a "physiological" and positive paracrine modulatory role in insulin secretion.

INGAP-PP can also increase β-cell mass, at least in experimental animal models; therefore, its administration could be a potential tool to treat people with either prediabetes or diabetes. This proposal is supported by both the encouraging results obtained in a previous clinical trial showing the beneficial effect of INGAP administration to people with type 1 and type 2 diabetes [17], and the stimulatory effect of INGAP-PP upon the in vitro differentiation of human ductal cells into islet-like cell clusters [31].

Although the current study was performed using hamster islets, the potential optimizing effect of INGAP-PP upon insulin secretion in

human islets while they are maintained in culture prior to their transplant might be worth testing.

Conflict of interest

We declare that no part of the work has been published before and that animal studies have been reviewed by the appropriate ethics committee. All funding sources supporting the work and all institutional or corporate affiliations are acknowledged, and none of the authors have commercial associations that might pose a conflict of interest in connection with the submitted article.

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