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REVIEW ARTICLE

IGF-1 Gene Therapy as a Potentially Useful Therapy for Spontaneous Prolactinomas in Senile Rats

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ARTICLE HISTORY

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Abstract: ***Background*:** Insulin-like growth factor1 (IGF1) is a powerful neuroprotective molecule. We have previously shown that short-term hypothalamic IGF1 gene therapy restores tuberoinfundibular dopaminergic neuron function in aging female rats.

***Objective*:** Our aim was to implement long-term IGF-I gene therapy in pituitary prolactinomas in senile female rats.

***Methods*:** Here, we assessed the long-term effect of IGF1 gene therapy in the hypothalamus of young (4 mo.) and aging (24 mo.) female rats carrying spontaneous pituitary prolactinomas. We constructed and injected a helper-dependent (HD) adenovector expressing the gene for rat IGF1 or the reporter red fluorescent protein DsRed. Ninety-one days post vector injection, all rats were sacrificed and their brains and pituitaries fixed. Serum prolactin (PRL), estrogen (E2) and progesterone (P4), as well as hypothalamic IGF1 content, were measured by RIA. Anterior pituitaries were immunostained with an anti-rat PRL antibody and submitted to morphometric analysis.

***Results*:** DsRed expression in the mediobasal hypothalamus (MBH) was strong after the treatment in the DsRed group while IGF1 content in the MBH was higher in the IGF1 group. The IGF1 treatment affected neither pituitary weight nor PRL, E2 or P4 serum levels in the young rats. In the old rats, IGF1 gene therapy reduced gland weight as compared with intact counterparts and tended to reduce PRL levels as compared with intact counterparts. The treatment significantly rescued the phenotype of the lactotropic cell population in the senile adenomas.

***Conclusion*:** We conclude that long-term hypothalamic IGF1 gene therapy is effective to rescue spontaneous prolactinomas in aging female rats.

Keywords: Aging, Hypothalamus, Pituitary, Adenomas, PRL, IGF-1, Gene therapy.

1. INTRODUCTION

In female, but not male rats, aging brings about a significant increase in the prevalence of prolactin (PRL)-secreting adenomas, chronic hyperprolactinemia and consequently, mammary tumors [1, 2]. The incidence of these pathologies begins to rise after the first year of life [3] when most females are found in constant estrus [4, 5]. They are typically characterized by extended periods of vaginal cornification and sustained estrogen secretion [6, 7]. It has been hypothesized that in female rats, continuous exposure to moderately high or medium levels of estrogens unopposed by progestagens leads to irreversible tuberoinfundibular dopaminergic (TIDA) neuron damage and degeneration, which is thought to be the main mechanism leading initially, to increased PRL secretion and later to the development of PRL-secreting pituitary adenomas [8, 9].

In a previous study, we reported that the Insulin-like growth factor I (IGF-I) gene therapy demonstrates efficacy for the short-term treatment of estrogen-induced prolactinomas in rats. The study also reported the effect of IGF-I gene therapy on pituitary lactotroph morphology [10] In a previous study, we reported that short-term (17 days) intrahypothalamic insulin-like growth factor 1 (IGF-1) gene therapy in old female rats restores the TIDA neuron population and reduces circulating PRL levels in senile female rats [11]. We did not assess the effect of the treatment on anterior pituitary morphology or mass. Neither did we know whether the restorative action of IGF-1 was transient or long-lasting. Bene-

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fiting from the availability of helper-dependent adenovectors, we were now able to implement long-term IGF-1 gene therapy in the hypothalamus of old female rats and assess the impact of the treatment on pituitary morphometry, mass and serum PRL levels.

2. MATERIALS AND METHODS

2.1. HD Adenovectors

Helper-dependent recombinant adenovectors (HD-RAds) were constructed using a kit sold by Microbix Biosystems (Ontario, Canada). The kit provides shuttle plasmid pC4HSU, helper virus H14 and the 293 Cre4 cell line. The construction procedure followed the guidelines of the Microbix manual and those described by Oka and Chan [12]. Briefly, an expression cassette containing either the cDNA for rat IGF-1 or the gene for the red fluorescent protein DsRed was cloned in pC4HSU, a plasmid that consists of the ITRs for Ad 5 virus, the packaging signal and part of the E4 adenoviral region plus a stuffer noncoding DNA of human origin which keeps a suitable size (28-31 Kbp) of the viral DNA so that it is efficiently packaged into capsids during vector generation but bands at sufficient distance from helper virus H14 in CsCl gradients, thus minimizing the risk of contamination of the newly generated vector. The shuttle vectors harboring the expression cassette of interest were transfected in 293 Cre4 cells which were then infected with the helper Ad H14 whose packaging signal is flanked by loxP sites recognized by the Cre recombinase expressed by the 293 Cre4 cells. Therefore, the helper virus provides in trans all of the viral products necessary for generation of the desired HD-RAd. Following iterated coinfections with the HD-RAd and H14 virus, a sufficiently high concentration of each HD-RAd was generated whereas very low levels of H14 were produced due to the cleavage of the packaging signal of H14 effected by the Cre recombinase. Both transgenes were under the control of the mouse cytomegalovirus promoter. The final adenovectors expressing either IGF-1 or DsRed were termed HD-RAd-IGF-1 and HD-RAd-DsRed, respectively.

The vectors were purified by ultracentrifugation in CsCl and then titrated and aliquoted. Titres were, 2.1X 10^{10} physical viral particles (PVP)/ml for the DsRed vector and 2.8 X 10^{10} for the IGF-I vector.

2.2. Animals

Forty-five young (4 mo.) and 45 senile (24 mo.) female Sprague-Dawley (SD) rats were used. Animals were housed in a temperature-controlled room (22 ± 2ºC) on a 12:12 h light/dark cycle. Food and water were available ad libitum. All experiments with animals were performed in accordance with the Animal Welfare Guidelines from the NIH (INIBIOLP's Animal Welfare Assurance No A5647-01) and approved by our IACUC (Protocol # T09-01-2013).

2.3. Surgical Procedures

Young and senile females were allotted to an intact, control or experimental group, thus forming 6 groups of 15 rats each: Young intact (Y-int), young control (Y-DsRed), young experimental (Y-IGFI), senile intact (S-int), senile control (S-DsRed) and senile experimental (S-IGFI). On experimental day 90, a small blood sample (0.3-0.4 ml) was taken from the tail veins of all rats. Serum was obtained and kept at -20 ºC for hormone assay.

On Experimental day 0, control and experimental animals received bilateral 2-µl intrahypothalamic injections containing 4 x 10^9 viral particles HD-RAd-DsRed or HD-RAd-IGF-1, respectively. For this purpose, rats were anesthetized by injection of xylazine (8 mg/kg, i.m.) and ketamine hydrochloride (40 mg/kg, i.p.) and placed in a stereotaxic frame. In order to access the arcuate-periventricular (ARC-PeV) region, the tip of a 26 gauge needle fitted to a 10 µl syringe was brought to the following coordinates relative to the bregma: 10.0 mm ventral, 3.0 mm posterior and 0.6 mm right and left [13].

On experimental day 91, eight rats from each group were sacrificed by quick decapitation, the brain was immediately removed from the cranium, placed on a dry ice block and the medial basal hypothalamus (MBH) dissected and homogenized for determination of IGF-1 content. The adenohypophyses were also removed, weighed and fixed in Bouin's fixative for 4 h. The remaining animals were placed under deep anesthesia and perfused with a fixative consisting of phosphate buffered formaldehyde 4%, (pH 7.4). Afterwards, each brain was removed and trimmed down to a block containing the whole hypothalamus. The block was then serially cut into coronal sections 40 µm thick on a vibratome.

2.4. Histology-Immunohistochemistry

Briefly, fixed pituitary glands were embedded in paraffin. Serial sections of 4 µm were obtained from the blocks following a ventral-to-dorsal sequence. The gland sections were immunostained and incubated for 1 h at room temperature with a primary anti-PRL antibody (Dako, CA, USA) diluted 1:100. Thoroughly washed sections were treated for 30 min with an EnVision reaction system (Dako, CA, USA). The chromogen diaminobenzidine was used. The specificity of the primary antibodies was monitored by blocking the immunocytochemical reaction after preabsorption with an excess of a related antigen [14].

2.5. Anterior Pituitary Morphometry

Morphometry was performed as described before [15]. Measurements of immunostained lactotropic cells were made with an image-analysis system (Imaging Technology, Optimas 5.2, USA). The number of cells per reference area (RA) was assessed in each field on an average of ten micrographs taken from two levels of the gland. These measurements were automatically recorded and processed, with the following parameters being calculated: cell size (CS, expressed in μm^2), volume density (VD = Σ cell area/RA) and cell density (CD = number of cells/RA). RA represents the total area throughout which the cells were scored. This area divided into the sum (Σ) of the individual cell areas (A) yielded VD, a parameter that represents an estimate of cell density according to accepted criteria. For morphometric analysis, 100 cells were recorded in each field.

2.6. Hormone Determination

Blood for hormone level determination was obtained from random cycling females, in the young groups. IGF-1

was extracted from the MBH by acid-ethanol cryoprecipitation and was radioimmunoassayed (RIA) using antibody UB2-495 distributed by Dr. AF Parlow, Pituitary Hormones and Antisera Center, UCLA Med. Center, LA. A. Recombinant human IGF-1 (rh IGF-1, Chiron Corp., Emeryville, California**)** was utilized as a tracer and unlabeled ligand. Intra and inter-assay coefficients of variation were 7.2 and 12.8%, respectively. Serum PRL was determined by RIA using the mouse materials provided by Dr. A. F. Parlow, Serum PRL was expressed in terms of rPRL RP-3. Coefficients of variation intra- and inter-assay for PRL ranged between 8.4% and 12.3%, respectively.

Serum E2 concentrations were determined by an RIA previously described [16]. Serum P4 levels were measured using a commercial RIA kit (Immunotech Laboratories, Inc. Glendale, CA, USA) [17]. Coefficients of variation intra- and inter-assay for both RIAs ranged between 4% and 6% and 8% and 12%, for E2 and P4, respectively.

2.7. Statistics

Analysis of the variance (ANOVA) was used to evaluate group differences. Duncan's method was chosen as a post hoc test.

3. RESULTS

Transgene expression in the hypothalamus was long-lived after injection of the HD-adenovectors. There was strong red fluorescence in the injection sites and in the ependymal cell layer of the third ventricle, 91 days post-HDRAd-DsRed treatment (Fig. **1**). Hypothalamic IGF-1 tissue content was significantly higher in young and old rats treated with HDRAd-IGF-1 as compared with intact counterparts (Table **2**).

As expected, pituitary weight was higher in the old than in the young intact rats. The same was true for the DsRed groups. Although for the IGF-1 group pituitary weight was not significantly higher in the aged *versus* young animals, there was a trend towards a higher weight in the aged pituitaries (Fig. **2A**).

Serum PRL levels were significantly higher in the senile rats for all three groups as compared with their young counterparts. However, the senile IGF-1 group showed a trend ($p=0.063$) towards a lower hyperprolactinemia than the intact senile group (Fig. **2B**). Serum E2 and P4 levels were not affected by IGF-1 gene therapy (Fig. **2 C&D)**. Immunohistochemical analysis of the pituitary glands revealed that in the senile rat's prolactinomas were frequent, in some cases showing chromophobic islands (Fig. **3 G** and **H**). IGF-1 gene therapy clearly rescued anterior pituitary gland morphology and lactotrophic cell distribution in the old glands (Fig. **3 K** and **L**). As expected, serum IGF-1 levels were lower in the aging females, being comparable in intact and IGF-1 rats (data not shown).

Morphometric analysis revealed that in the young rats IGF-1 gene therapy does not have a major impact. In contrast, IGF-1 gene therapy in old rats reduced the number of PRL cells per 1,000 μm^2 to youthful values, while cell surface was increased to normal values. Cell density and volume density, where further reduced by the treatment (Table **1**).

4. DISCUSSION

Treatments like surgery, radiotherapy and pharmacology have led to significant advances in the treatment of pituitary prolactinomas. Nevertheless, none of them or their combination constitutes a definitive solution for this pathology [18]. This has led researchers to seek alternative approaches like gene therapy which emerges as a potentially suitable therapeutic alternative. Initially, it was found that a herpes simplex virus type-1 (HSV1)-derived vector was effective in *in vivo* gene therapy approaches in rats carrying E2-induced prolactinomas [19]. Subsequently, an adenovector termed RAdTK, which harbors the HSV-1 thymidine kinase (TK) suicide gene under the control of the cytomegalovirus (CMV) promoter, was employed to transfer the TK gene to GH_3 and AtT_{20} pituitary tumor cells. When the prodrug ganciclovir (which after phosphorylation by HSV-1 TK becomes toxic) was added to cultures of RAdTK-treated GH_3 and AtT_{20} cells, substantial cell destruction was observed [20]. In the same study, stereotaxic injection of RAdTK to estrogen/sulpiride-induced rat prolactinomas, followed by two

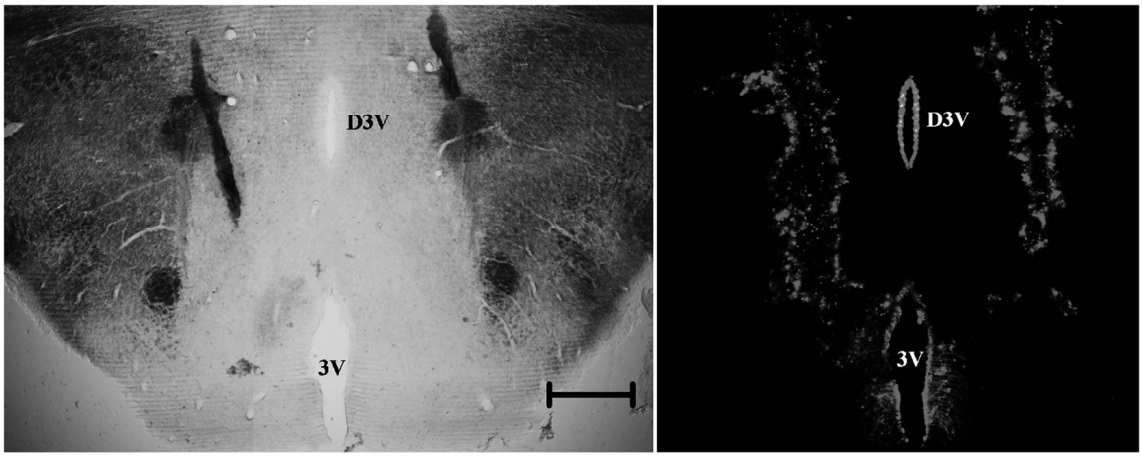

Fig. (1). Expression of Ds-Red in the hypothalamus of a senile female rat after HDRAd-DsRed injection. Rats were sacrificed 91 days after receiving a single bilateral intrahypothalamic injection of HDRAd-DsRed. Right panel shows expression of DsRed in hypothalamic sections. The left panel shows a bright field view of the same section. For further technical details see M&M.; 3V: third ventricle. D3V; Scale bar, 100 μm.

Table 1. Morphometry of the lactotroph population in young and old rats receiving gene therapy.

Young	Intact	HDRAd-DsRed2	HDRAd-IGF-1
VD (volume density)	92.9 ± 4.0	84.7 ± 5.1	83.5 ± 1.2
CD (cell density)	292.5 ± 32.1	285.8 ± 20.1	305.2 ± 25.2
CS (cell size)	35.5 ± 2.1	26.2 ± 0.5*	27.6 ± 2.1 *
N° PRL cells/1,000 µm^2	30.3 ± 2.3	27.2 ± 0.4	31.0 ± 3.1
Old			
VD	47.6 ± 3.1	22.3 ± 2.3*	11.3 ± 1.0 **
CD	227.0 ± 12.2	101.5 ± 8.2**	98.6 ± 4.2 **
CS	21.7 ± 2.1	31.6 ± 4.2 *	31.0 ± 4.3 *
N° PRL cells/1,000 µm^2	47.7 ± 2.1	36.7 ± 6.1	26.5 ± 4.1**

Data are expressed as mean ± SEM. Asterisks represent the level of significance regarding intact counterparts; * $p < 0.05$; ** $p < 0.01$. One-way ANOVA followed by Duncan *post hoc* tests for comparing pairs of means. N=5.

daily doses of 25 mg ganciclovir/kg for a week caused a significant reduction of tumor size and circulating PRL [20]. Another type of gene therapy strategy for the treatment of pituitary cancer is based on the transfer of a gene able to rescue the normal phenotype of pituitary tumor cells constitutes another gene therapy approach. The approach was used in mice heterozygous for the retinoblastoma (RB) tumor suppressor gene ($Rb^{+/-}$ mice) which develop pituitary intermediate lobe melanotrope tumors [21].

Our group has reported that IGF-1 gene therapy in estrogen-induced rat pituitary tumors (intrapituitary injection of the adenovector) reduced hyperprolactinemia and induced a partial rescue of the lactotroph population phenotype [10].

It has been long-known that aging female rats undergo a progressive dysfunction of the hypothalamic TIDA neurons [22], a process that can be partially reversed by short-term hypothalamic gene therapy [11]. In the present study we show that a single intrahypothalamic injection of a helper-dependent adenoviral vector harboring the rIGF-1 cDNA allows long-term IGF-1 expression and significantly rescues the morphometric parameters of the lactotroph population. The treatment reduces gland size and attenuates chronic hyperprolactinemia. It is likely that some transgenic IGF-1 injected into the hypothalamus leaked to the pituitary gland. Since intrapituitary IGF-1 has been shown to be beneficial for the treatment of pituitary adenomas [10], the possibility exists that the present results represent a two-fold action of the peptide on the adenomas, namely, a restoration of the dysfunctional TIDA neuron population and a direct action on pituitary tissue. The former hypothesis implies that a substantial number of dysfunctional TIDA neurons (TH negative) remain in the hypothalamus of old female rats and that these neurons can be reactivated. This idea is supported by a study in young female rats which were i.m. injected with estrogen. As expected the animals developed large prolactinomas and TIDA neurons were fully inhibited (no tyrosine hydroxylase expression). However, when on experimental day 21, E2 injections were discontinued and rats let live until 12 months of age, TH-producing neurons reappeared in the arcuate nucleus and at the same time, pituitary size and hyperprolactinemia were markedly reduced [23]. The P4 and E2 data indicate that the improvement induced by long-term IGF-1 gene therapy does not stem from an effect on ovarian steroid levels.

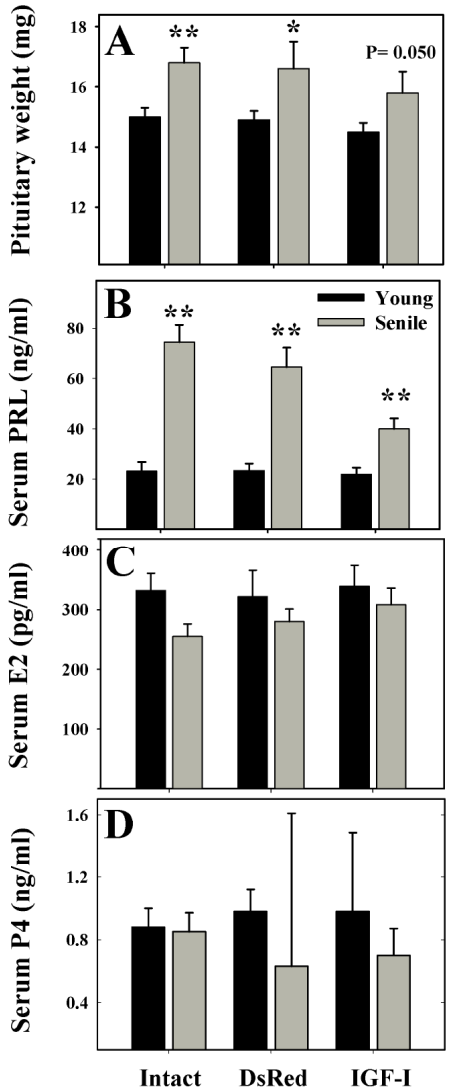

Fig. (2). Pituitary weight and serum PRL, E2 and P4 levels in young and old rats. Blood samples were taken at the end of the experiment. Asterisks over bars indicate a significant difference *versus* the corresponding young counterparts. *, P<0.05; **, P<0.01. N=8 for young and 7 for old rats.

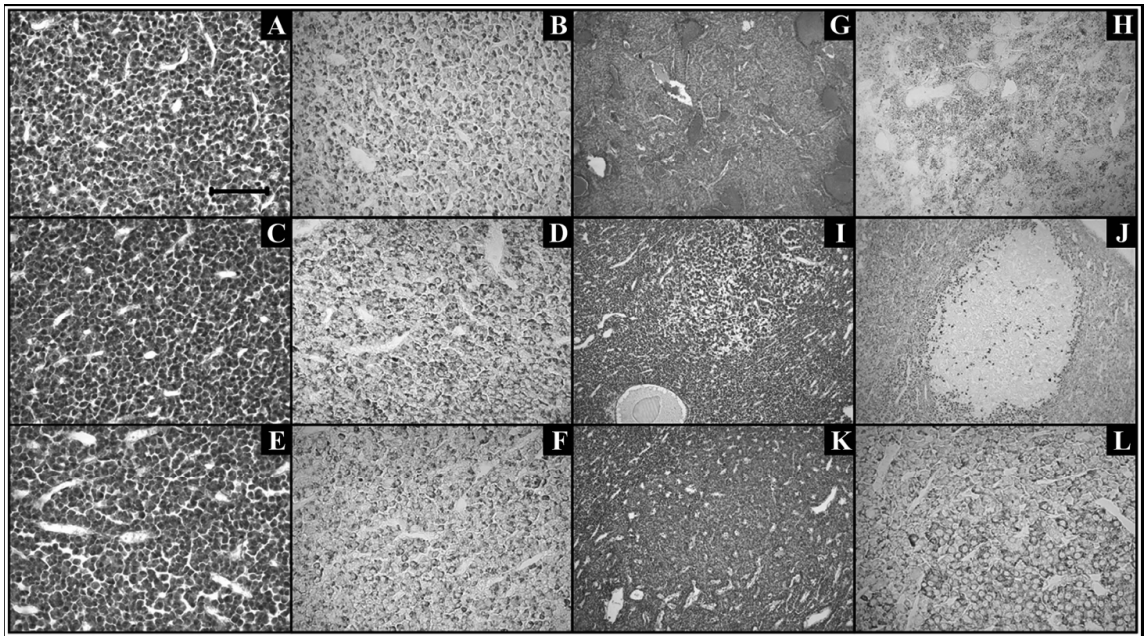

Fig. (3). Lactotoph cell population in young and old female rats. A-B, Young intact. C-D, Young HD-RAd-DsRed. E-F. Young HD-RAd-IGFI. G,H, old intact. I-J. old HD-RAd-DsRed. K-L.Old HD-RAd-IGF-1. Panels A, C, E, G, I, K are H&E stainings, panels B,D, F, H, J, L correspond to immunohistochemical staining using peroxidase, anti-PRL. Scale bar= 100 μm.

Table 2. Hypothalamic IGF-I content (pg/mg tissue) in young and old rats receiving gene therapy.

Eptl. Group	Intact	HDRAd-DsRed2	HDRAd-IGF-1
Young	77 ± 11	96 ± 13	125 ± 12**
Old	60 ± 11	84 ± 12	120 ± 11**

Data are expressed as mean ± SEM. Asterisks represent the level of significance regarding intact counterparts; ** $p < 0.01$. One-way ANOVA followed by Duncan *post hoc* tests for comparing pairs of means. N=8 for young and 7 for old rats.

CONCLUDING REMARKS

Taken together, the body of data available from us and others, points to a restorative action of IGF-1 on pituitary prolactinomas, both at the pituitary and hypothalamic level. Since HD adenovectors are neither immunogenic nor oncogenic, they are safe for use in human patients. Besides, they allow long-term expression of transgenes. In this context, a possible therapeutic scenario for pituitary prolactinomas envisions that immediately after tumor removal, the surgeon administers an IGF-1 HD adenovector into the pituitary cavity in an attempt to prevent residual lactotropic cells from growing a new adenoma.

Nevertheless, it should be pointed out that a major limitation of the gene transfer systems so far developed is the difficulty in obtaining clinically relevant levels of gene expression. Moreover, no single vector has emerged as being optimal for all applications. Current systems differ in their suitability for *ex vivo versus in vivo* adoption, and their capability for persistent gene expression. Before clinical trials can be conducted, more preclinical studies are necessary. Future studies employing "omics" technology would help better understand the underlying mechanisms, as adapted from previous studies [24].

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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