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Rapid paper

Rat Humanin is encoded and translated in mitochondria and is localized to the mitochondrial compartment where it regulates ROS production

Vladislava Paharkova [a, 1], Griselda Alvarez [a, 1], Hiromi Nakamura [a], Pinchas Cohen [b], Kuk-Wha Lee [a, *]

[a] Pediatric Endocrinology, Mattel Children's Hospital UCLA; David Geffen School of Medicine at UCLA, USA
[b] Davis School of Gerontology, University of Southern California, USA

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ABSTRACT

Evidence for the putative mitochondrial origin of the Humanin (HN) peptide has been lacking, although its cytoprotective activity has been demonstrated in a variety of organismal and cellular systems. We sought to establish proof-of-principle for a mitochondria-derived peptide (MDP) in a rat-derived cellular system as the rat HN sequence is predicted to lack nuclear insertions of mitochondrial origin (NUMT). We found that the rat HN (Rattin; rHN) homologue is derived from the mitochondrial genome as evidenced by decreased production in Rho-0 cells, and that peptide translation occurs in the mitochondria as it is unaffected by cycloheximide. Rat HN localizes to the mitochondria in cellular subfractionation and immunohistochemical studies. Addition of a HN analogue to isolated mitochondria from rat INS-1 beta cells reduced hydrogen peroxide production by 55%. In summary, a locally bioactive peptide is derived and translated from an open reading frame (ORF) within rat mitochondrial DNA encoding 16S rRNA.

1. Introduction

Humanin (HN) is a 24 amino acid putative mitochondrial-derived peptide (MDP) encoded within an open reading frame (ORF) found in the mitochondrial 16S ribosomal RNA (Capt et al., 2015; Hashimoto et al., 2001; Maximov et al., 2002). Antibodies were generated against the predicted peptide (Ikonen et al., 2003; Tajima et al., 2002), an ELISA developed (Chin et al., 2013), and HN levels assayed in a variety of biological fluids (Muzumdar et al., 2009). Since its discovery, studies have revealed diverse cytoprotective effects of HN against various cellular stressors in multiple organ systems, including recently described protective properties against chemotherapy-related side effects (Eriksson et al., 2014; Jia et al., 2015). In addition, HN improves survival of pancreatic beta cells and delays the onset of diabetes (Hoang et al., 2010); it also improves peripheral insulin sensitivity via a central mechanism (Muzumdar et al., 2009); and it increases glucose-stimulated insulin secretion (Kuliawat et al., 2013).

Perhaps the greatest amount of work that has been published on HN is its characterization as a neurosurvival factor capable of antagonizing Alzheimer's disease-related cellular insults (Hashimoto et al., 2001). Proposed mechanisms of action for HN include antagonistic effects on two mitochondrially-localized pro-apoptotic factors, insulin-like growth factor binding protein-3 and Bax (Guo et al., 2003; Ikonen et al., 2003).

Humanin-induced signaling has been demonstrated to occur via a trimeric cell surface receptor comprised of CNTFR, WSX-1, and gp130 (Hashimoto et al., 2009) that activates multiple signaling cascades including STAT3 (Hashimoto et al., 2005). In addition, the FPR-like-1 (FPRL1) receptor, which is a member of the G protein-coupled formylpeptide receptor (FPR) family that activates the ERK pathway has been proposed to be a functional receptor for HN action (Harada et al., 2004). These data suggest that HN may have a multiplicity of mechanisms of action.

The origin of the HN gene remains elusive as it is still unclear whether HN is translated in the mitochondria or the cytoplasm. Given that the mitochondrial genetic code for HN differs from the cytoplasmic genetic code, translation in the mitochondria would

* Corresponding author. Division of Pediatric Endocrinology, Mattel Children's Hospital at UCLA, David Geffen School of Medicine at UCLA, 10833 Le Conte Ave., MDCC 22-315, Los Angeles, CA 90095 USA.
E-mail address: kukwhalee@mednet.ucla.edu (K.-W. Lee).
[1] VP and GA contributed equally to this work.

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result in a shorter peptide (Bodzioch et al., 2009). Conversely, HN could originate from the nuclear genome (Guo et al., 2003). There are 13 nuclear loci that maintain the open reading frames of 15 distinct full-length HN-like peptides, and at least ten of them may be functional genes (Bodzioch et al., 2009). These sequences could be classified as nuclear insertions of mitochondrial origin (NUMTs) (Ramos et al., 2011). NUMTs are fragments of mitochondrial DNA that have been integrated into the nuclear genome throughout the evolutionary process by a potential mechanism involving transposable elements (Mishmar et al., 2004). There are hundreds of NUMTs in the human genome, and they come in various sizes with varying degrees of homology with the original sequences (Mishmar et al., 2004; Mourier et al., 2001). Nuclear HN NUMTs have also been found in other species; however, rats do not possess any coding nuclear isoforms for HN (Bodzioch et al., 2009), making rat-derived cells an attractive model to study the origin of the rat HN (Rattin; rHN) homologue. In our work, we identify the origin and translation site of rHN.

2. Results/discussion

Analysis of HN-like gene sequences in rodents revealed that rats apparently contain no coding nuclear HN genes but only a mitochondrially-encoded HN (Bodzioch et al., 2009). As an initial step in the identification of the origin and translation site of rHN, INS-1 cells were treated for 4 weeks with ethidium bromide (EtBr) to deplete them of mitochondrial DNA (mtDNA) as previously described (Miller et al., 1996). INS-1 cells treated with EtBr showed an expected reduction in COX I (a protein of mitochondrial genomic origin) by Western blot. rHN peptide was not detected in these mtDNA-depleted (Rho-0) cells (Fig. 1).

Translation localization of rHN was achieved through treatment of INS-1 rat insulinoma cells with the translation inhibitors chloramphenicol (mitochondria-specific), and cycloheximide (cytoplasmic-specific). Translation inhibition was confirmed by measuring levels of COX I, a respiratory chain protein translated in the mitochondria, and UQCRC2 (ubiquinol-cytochrome c reductase core protein II), a nuclearly-encoded respiratory chain protein that is transported into the mitochondria after cytoplasmic translation.

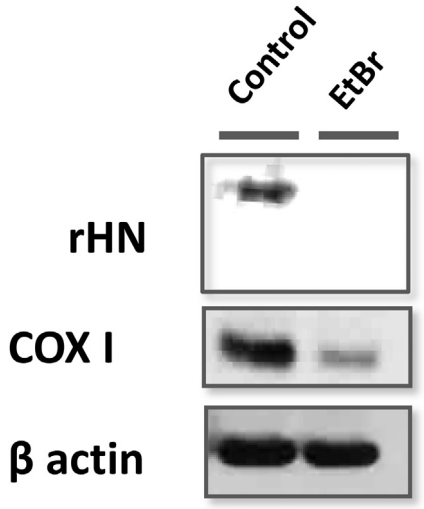

Fig. 1. rHN is derived from the mitochondrial genome. INS-1 cells were treated with ethidium bromide (EtBr) for 60 days. EtBr-induced loss of mitochondrial DNA was confirmed by decreased cytochrome C oxidase subunit I (COX I) signal. rHN was present in untreated INS-1 cells. However, after EtBr treatment, rHN was not expressed in cells that were depleted of mitochondrial DNA.

In INS-1 cells treated with chloramphenicol the rHN peptide signal decreased over time as detected by Western blot. However, rHN signal was preserved in cells treated with cycloheximide, suggesting mitochondrial translation (Fig. 2). Rat L6 myoblasts demonstrated a similar pattern (data not shown).

To identify the subcellular location of rHN peptide in insulin-producing beta cells, we analyzed subcellular fractions of INS-1 rat insulinoma cells by Western blot utilizing a polyclonal antibody against rHN. rHN was not detected in the microsomal fraction. rHN was found in fractions containing mitochondria as evidenced by co-localization with the mitochondrial peptides Hsp60 and cytochrome C oxidase subunit I (COX I); additionally, rHN co-localizes with the pro-apoptotic Bax (Fig. 3), a previously described HN binding-partner (Jia et al., 2013). Therefore, rHN peptide localizes to mitochondria in INS-1 cells. The apparent molecular weight difference in the synthesized rHN peptide and endogenous rHN may be due to post-translational modifications of the endogenous peptide. Clearly, sequencing of the endogenous peptide from various species will definitively answer many questions re: origin, translation location, and post-translation modifications.

Supporting the mitochondrial localization of HN, previous work has shown that HN localizes to the midpiece below the head of individual human sperm (Moretti et al., 2010), an area composed of a central filamentous core with many mitochondria spiraled around it, used for ATP production (Fig. 4A). In rats, rHN has been localized to various individual testicular cells, including Leydig cells, spermatocytes, and spermatids (Colon et al., 2006). Although mouse HN (mHN) has previously been detected in mouse testicular tissue (Tajima et al., 2002) we show via immunohistochemistry under 1000× magnification that mHN is detected at the midpiece of sperm, suggesting mitochondrial localization (Fig. 4B). mHN was also detected in the nucleolar regions of Sertoli cells and/or spermatogonia, visible at the basal membrane of the seminiferous tubules and nucleolar regions of cells outside the seminiferous tubules, comprised mainly of Leydig cells.

Recent studies have shown that HNG, a potent HN analog, induces a rapid and sustained activation of antioxidant defense systems and attenuates oxidative stress in rat cardiac myoblasts exposed to hydrogen peroxide (H_2O_2) (Klein et al., 2013). Though pancreatic beta cells have low antioxidant activity at baseline (Drews et al., 2010), an HNG-induced increase in glucose oxidation rate that results in increased ATP and increased production of reactive oxygen species (ROS), did not disrupt mitochondrial membrane integrity (Kuliawat et al., 2013). This finding suggests a role of HN in the regulation of oxidative stress processes in the beta cell. Therefore, we hypothesized that the cytoprotective properties of HN may be due to its ability to directly attenuate the production of mitochondrial ROS and thereby decrease oxidative stress of the cell. In order to exclude the effects of other antioxidant mechanisms present in the cell, we measured the direct effect of HNG addition on H_2O_2 production from isolated INS-1 mitochondria and show a 55% reduction in H_2O_2 production (Fig. 4C).

Recent insights on the role of mitochondria in disease suggest that the mitochondria may serve as a potential target for novel therapeutic approaches. In Type 1 diabetes, it has been proposed that production of ROS by mitochondria during immune destruction acts as a feed-forward activation loop for apoptosis (Szabadkai and Duchen, 2009). In Type 2 diabetes the acquired metabolic alterations, including hyperglycemia, hyperlipidemia and hyperinsulinemia, can promote the production of ROS and damage mitochondria in insulin responsive tissues (Szendroedi et al., 2012). Recent reports demonstrate that HN induces physiologic changes including increasing insulin sensitivity and enhanced insulin secretion in beta cells (Kuliawat et al., 2013). To date, several studies

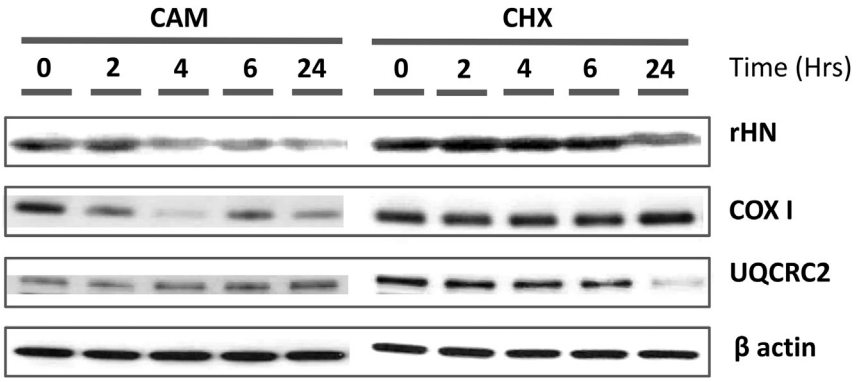

Fig. 2. rHN is translated in the mitochondria. rHN expression decreases over time with mitochondria-specific translation inhibitor chloramphenicol but does not change with cytoplasmic-specific translation inhibitor cycloheximide, suggesting that rHN is translated in the mitochondria. CHX: cycloheximide (50 μg/ml); CAM: chloramphenicol (100 μg/ml).

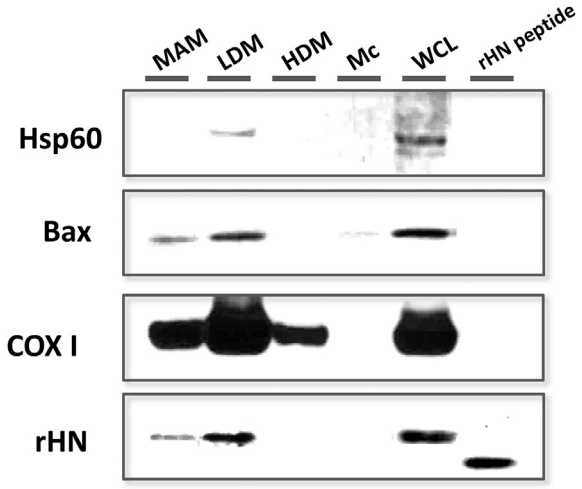

Fig. 3. rHN localizes to mitochondria. rHN localizes to cellular fractions that contain mitochondria, evidenced by co-localization with mitochondrial specific proteins Hsp60 (mitochondrial matrix), Bax (outer mitochondrial membrane), and COX I (inner mitochondrial membrane). MAM: mitochondrial associated membrane fraction; LDM: lower density mitochondrial fraction; HDM: higher density mitochondrial fraction; Mc: microsomes; WCL: whole cell lysate; rHN peptide: synthesized rHN peptide (20 ng).

have described important protective properties of HN against a variety of insults that reveal its potential for therapeutic applications, particularly in states of high oxidative stress (Yen et al., 2013). However, these effects have yet to be explained mechanistically. We propose that these effects could originate from antioxidant-like effects of HN that result in decreasing ROS. In particular, the localization of mHN to the mitochondria-rich spermatozoa midpiece may be a protective mechanism to the nucleic acid-rich head from the damaging byproducts of intense oxidative phosphorylation in the midpiece.

Though the sequence of the putative HN peptide is found in the mitochondrial genome that codes for 16S ribosomal RNA, no convincing evidence has previously been presented to substantiate the claim of its origin from this site. Although there is substantial evidence for putative coding nuclear short open reading frames (sORFs), the functionality of most of these peptide products is still under investigation (Andrews and Rothnagel, 2014).

Here we have demonstrated that the subcellular coding origin, translation, and localization of rHN are mitochondrial. However, we cannot rule out that HN may also be localized to other subcellular organelles with undiscovered specific functions. We have demonstrated that rHN is transcribed from a region previously thought to be non-translated from the mitochondrial genome. Definitive data regarding the primary peptide sequence of endogenous HN generated by MS/MS analysis will clarify mitochondrial vs. nuclear origin. Our data supports the idea that translation of this area results in a gene product that is not only involved in mitochondrial function but also possesses systemic bioactivity that others have described.

Importantly, a recent study describes a short ORF (sORF) within the mitochondrial 12S rRNA encoding a 16 amino acid peptide that regulates insulin sensitivity and metabolic homeostasis named MOTS-c (Lee et al., 2015). Like rHN, rat MOTS-c does not have any NUMT sequences, making mitochondrial DNA its exclusive source. Human MOTS-c translation likely occurs in cytoplasmic ribosomes, as the mRNA sequence contains an early stop codon when read using the mitochondrial/bacterial code. MOTS-c is measurable in rat plasma; however, subcellular localization of rat MOTS-c translation has not been formally investigated.

Thus, our work shows that mitochondrial-derived peptides (MDPs) have evolved to be important signaling molecules that can be translated in either mitochondrial (as in the case of rHN) or cytoplasmic (for human MOTS-c) ribosomes.

Pancreatic beta cell susceptibility to a variety of insults is the basis of the development of both Type 1 and Type 2 Diabetes Mellitus (Imai et al., 2013; Jezek et al., 2012; Potter et al., 2014). Accumulating data suggests that oxidative stress and dysregulated signaling result in cellular events that contribute to progressive dysfunction and ultimately cellular apoptosis of beta cells (Supale et al., 2012). Abnormal mitochondrial function has been documented in insulin resistance and diabetes progression (Evans et al., 2002). Strategies that reverse beta cell dysfunction and decrease oxidative stress hold great potential for improving outcomes and can potentially attenuate diabetes development or progression. Indeed, oxidative stress-induced mitochondrial dysfunction has been implicated in a variety of disease states, and addressing this dysfunction may have broader implications for other conditions.

In conclusion, a locally bioactive peptide is derived and translated from an open reading frame (ORF) within rat mitochondrial DNA encoding 16S rRNA. Attenuation of oxidative stress is likely a link to the cytoprotective effects of HN by its direct participation in cellular processes that produce ROS. Our findings demonstrate that HN is a novel type of antioxidant. These observations have potential implications in a variety of pathophysiologic conditions as well as the physiologic aging process. Clearly, additional investigation should be undertaken to elucidate the molecular mechanisms by which rHN and its human and mouse homologues exert their various bioactivities.

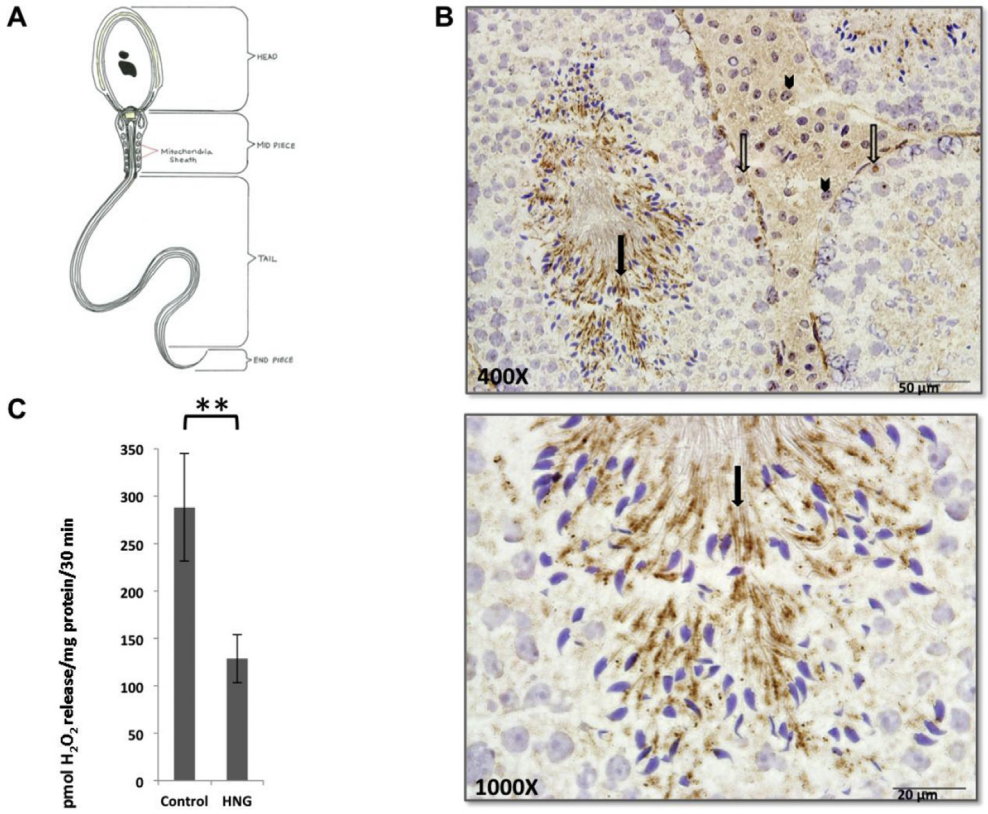

Fig. 4. mHN localizes to mitochondria-rich areas of spermatozoa. **(A)** Cartoon of spermatozoa. Highest density of mitochondria is located in the mitochondrial sheath of the mid-piece. **(B)** Immunohistochemical localization of HN in testes of 40-day-old mice. Black arrows indicate mitochondria in spermatozoa staining positively for mHN (400× (upper) and 1000× (lower) magnification). Open arrows show Sertoli cell and/or spermatogonia nucleolar staining for mHN. Arrowheads point to nucleolar staining for mHN in Leydig cells. **(C)** Direct attenuation of mitochondrial hydrogen peroxide production (H_2O_2) by HNG. HNG, an ultrapotent analogue of HN, was incubated with isolated mitochondria from INS-1 cells. A 55% reduction in H_2O_2 production compared to untreated controls was demonstrated. $^{**}p < 0.013$.

3. Materials and methods

3.1. Cell lines/reagents

Rat insulinoma cell line INS 832/13 (INS-1) was provided by Dr. C. Newgard (Durham, NC, USA). Cells were cultured (Hohmeier et al., 2000). Reagents, unless otherwise specified, were obtained from Sigma (St. Louis, MO, USA).

3.2. Subcellular fractionation

Membrane-associated fractions were isolated following the approach utilized by Bozidis et al. (Bozidis et al., 2007). This procedure combines differential centrifugation and a self-generating Percoll gradient centrifugation.

3.3. Western blot and antibodies

INS-1 cells were treated with cycloheximide (50 μg/ml) and chloramphenicol (100 μg/ml) for the times indicated. The cell monolayer was washed with PBS and lysed in RIPA buffer (Thermo Scientific, Rockford, IL, USA) with protease inhibitor cocktail. Protein concentration was determined (Bio-Rad protein assay, Bio-Rad, Hercules, CA, USA). Proteins were resolved by NuPage 4–12% Bis-Tris gel (Life Technologies, Grand Island, NY, USA) electrophoresis and electroblotted onto PVDF membranes (Millipore, Billerica, MA, USA). For immunodetection, membranes were blocked in 5% BSA in TBS with Tween-20 (TBST) and incubated in TBST with rattin antibodies (1:500; Phoenix Pharmaceuticals, Burlingame, CA, USA), cytochrome C oxidase subunit I (COX I) (monoclonal (mouse), Life Technologies, Grand Island, NY, USA), or UQCRC2 (polyclonal (chicken), GeneTex, Irvine, CA, USA). For chemiluminescent detection, blots were incubated with Peroxidase labeled Anti-Rabbit or Anti-mouse IgG secondary Antibody (1:20,000; cat# PI-1000 (Rabbit), PI-2000 (mouse), Vector Labs, Burlingame, CA, USA) at room temperature, followed by enhanced chemiluminescence detection (Immobilon Western HRP substrate Luminol Reagent, Millipore, Billerica, MA, USA).

3.4. Mitochondrial DNA depletion

To generate Rho-0 (r°) cells, INS-1 cells were treated with ethidium bromide (0.4 mg/ml) in RPMI 1640 supplemented with 100 mg/ml of sodium pyruvate (MP Biomedicals, Santa Ana, CA, USA) and 50 mg/ml of uridine. Cultures were maintained for 4 weeks, during which time r° status was monitored by western blot for COX I.

3.5. Immunohistochemistry

Immunohistochemistry was done on 5 μm sagittal and transverse stock 40 days-old mouse testes cryosections obtained from the Translational Pathology Core Laboratory (TPCL) UCLA. Tissue fixation was performed with 10% buffered formalin-phosphate for 30 min at RT, followed by antigen retrieval for 20 min at 95–99 °C with Tris-EDTA antigen retrieval buffer pH 9.0 (Abcam, Cambridge, MA, USA). Blocking Buffer–Protein Block–serum free (Dako, Carpinteria, CA, USA) was used for 60 min at RT. The primary antibody (IgA column-purified polyclonal rabbit anti-human HN antibody

(Ikonen et al., 2003)) was applied at a final concentration of 7 μg/mL and incubated at 4 °C overnight. Sections were washed and incubated with VECTASTAIN® Elite ABC Reagent with ImPACT DAB Peroxidase Substrate (Vector Labs, Burlingame, CA, USA). Nuclear counterstain was done with Hematoxylin QS (Vector Labs, Burlingame, CA, USA).

3.6. Measurement of hydrogen peroxide production

Measurement of hydrogen peroxide (H_2O_2) production was performed on isolated mitochondria from INS-1 cells. Intact mitochondria were obtained using the Qproteome Mitochondria Isolation kit (Qiagen, Valencia, CA, USA). Samples were kept at 4 °C at all times and experiments performed within 5 h of mitochondria isolation. Mitochondrial protein concentration was measured by the Bradford method using bovine serum albumin as a standard.

Mitochondrial H_2O_2 release was determined with the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Grand Island, NY, USA). Mitochondria were incubated with 1 μM HNG followed by the Amplex Red assay that was conducted for 30 min at 37 °C in 96-well plate format. H_2O_2 release was determined from a positive control, generated by hydrogen peroxide. Released H_2O_2 was measured spectrophotometrically at 560 nm absorbance.

3.7. Statistical analysis

Data are expressed as means ± SD. Differences between groups were compared by two-tailed Student's *t*-test. $p < 0.05$ was considered significant.

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