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Profiling of heat shock proteins 27 and 70 in adenoids of children

Hyun Jin Min[1,2] · Joon Soon Park[1] · Chang Eurn Kim[1] · Kyung Soo Kim[1]

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Abstract

Purpose Heat shock protein (HSP)27 and 70 are molecular chaperones that may have immunomodulatory functions. We determined if and at what levels each are expressed in the adenoids of pediatric subjects. We also examined tissue distributions, associated clinical characteristics, and antibacterial effects.

Methods Western blot, enzyme-linked immunosorbent assay (ELISA), and immunohistochemistry were applied to adenoidal tissues and lavage fluids obtained from children ($N=40$) undergoing adenotonsillectomy.

Results Via western blot and ELISA, both HSP27 and 70 were regularly detected in adenoidal tissue and in lavage fluid samples. HSP27 was highly expressed in epithelium, whereas HSP70 showed strong subepithelial positivity and bore a significant relation to adenoidal size. Assayed levels of HSP27 and 70 correlated inversely, and their addition to culture media independently increased bacterial numbers (*Staphylococcus aureus*). Upon the precipitation of each from adenoidal lavage fluids, bacterial counts declined.

Conclusions HSP27 and 70 are readily expressed in the adenoids of children and may be implicated in immunologic responses.

Keywords Adenoid · Heat shock protein · HSP 27 · HSP 70

Introduction

The adenoids (nasopharyngeal tonsils), accompanied by palatine, lingual, and tubal tonsils, are part of Waldeyer's tonsillar ring [1]. Anatomic positioning at the upper reaches of respiratory and digestive tracts ensures continuous adenoidal exposure to both airborne and gastrointestinal antigens [2]; and as a form of mucosa-associated lymphoid tissue, the adenoids are important for host defense, triggering immune reactivity. Structurally, they resemble lymph nodes. They also function similarly, acting as effector organs of adaptive immunity [3]. Adenoidal T cells are active in cytolysis and cytokine production, and the binding of secretory IgA (the chief antibody class of adenoids) to bacteria helps suppress bacterial colonization [4].

Unlike their much-probed ties to adaptive immunity, adenoidal contributions to innate immunity have received little attention. One particular study (aimed at expression of tight junctions and toll-like receptors) has implicated the lymphoepithelial component of adenoids in this regard [5]. The innate immune system acts to detect bodily perils and react by releasing host-derived mediators, currently termed damage-associated molecular patterns (DAMPs) [6]. In general, DAMPs exist within cells, where they exert well-defined effects in the absence of cellular stress [7]. Upon stimulation, however, they may be released extracellularly as innate and adaptive immune regulators [7].

Heat shock proteins (HSPs) are molecular chaperones that are grouped into families based on molecular weight (i.e., HSP110, HSP90, HSP/HSC70, HSP60, HSP40, and HSP10-30). They participate in proper protein folding and prevent aggregation of newly synthesized polypeptide chains within cells. Aside from such roles, they also behave as cytokines and cytoprotective agents in the extracellular milieu [8]. Ostensibly, they then qualify as DAMPs and are critical in immune response mechanisms [9].

✉ Kyung Soo Kim
99-21045@hanmail.net

[1] Department of Otorhinolaryngo-Logy-Head and Neck Surgery, Chung-Ang University Hospital, College of Medicine, 224-1 Heukseok-dong, Dongjak-gu, Seoul 156-755, Republic of Korea

[2] Chung-Ang University Hospital, Biomedical Research Institute, 224-1 Heukseok-dong, Dongjak-gu, Seoul 156-755, Republic of Korea

It is already known that the upper airway mucosa contains DAMPs, such as HMGB1 and HSPs. In fact, HSPs are reportedly highly expressed in the upper airway [10–12]. The present study was conducted to evaluate HSP27 and 70 expression levels/patterns in adenoidal tissue and examine associated clinical characteristics. The roles of these proteins in inflammatory responses to bacterial infection were also preliminarily assessed.

Methods

Study participants

This study was approved by the Institutional Review Board of Chung-Ang University College of Medicine (C2015092[1550]), obtaining informed consent from each participant. A total of 40 children (boys 20; girls 20; mean age 4.97 years; range 3–7 years) were enrolled (Table 1), each undergoing adenotonsillectomy for upper airway obstruction and sleep disorders between July 2015 and March 2016 at the Department of Otorhinolaryngology of Chung-Ang University Hospital (Seoul, Korea). Adenoidal size was evaluated by paranasal sinus (PNS) X-rays [13]. None of the subjects had congenital anomalies or systemic diseases, and use of any medication 2 weeks prior to surgery was grounds for exclusion.

Fluid and tissue collection

Adenoidal secretions were obtained as in our previous protocol, with minor modifications [10]. Briefly, the surfaces of adenoids were bathed in normal saline solution (5 mL), applied via 30° endoscope, retrieving approximately 4 mL after 3 s. Once adenoidal secretions were sampled, adenoid cutting forceps were used (prior to adenoidectomy) to acquire tissue (~0.5 × 0.5 cm) for immediate storage (−70 °C).

Table 1 Clinical characteristics of study participants ($N = 40$)

Characteristic	Value
Sex	
Female	20
Male	20
Age (years)	4.97 ± 0.91 (3–7)
Body mass index (kg/m^2)	16.32 ± 3.00
Symptom duration (mo)	21.52 ± 7.23 (2–36)
Adenoidal size (mm)	20.38 ± 4.83 (4.21–28.95)

Data expressed as *N* or mean ± standard deviation (range)

Western blot analysis

The tissue samples were lysed in RIPA lysis buffer incorporating various protease inhibitors (Sigma-Aldrich, St. Louis, MO, USA). Thereafter, protein samples (30 μg) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for transfer to nitrocellulose membranes. Primary antibodies to HSP27, HSP70 (Abcam, Cambridge, UK), and β-actin (Santa Cruz Biotechnology, Dallas, TX, USA) were then applied, followed by sequential incubation in HRP-labeled goat anti-rabbit or anti-mouse immunoglobulin (Jackson Laboratory, Bar Harbor, ME, USA) secondary antibodies. Signals were detected through enhanced chemiluminescence (Amersham, Little Chalfont, UK), using Image J software (National Institutes of Health [NIH], Bethesda, MD, USA) to quantify relative band intensities.

HSP quantification in adenoidal lavage fluids

Levels of secreted HSP27, HSP70, and tumor necrosis factor alpha (TNF-α) in adenoidal lavage fluids were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) as instructed by manufacturer.

Immunohistochemical analysis

Formalin-fixed, paraffin-embedded adenoidal tissue was sectioned and affixed to glass slides. Incubation (10 min) in sodium citrate buffer solution (pH 6.0, 0.01 M) was first required for antigen retrieval. After blocking of nonspecific binding sites, the sections were incubated overnight at 4 °C with primary antibodies to HSP27 or 70. Secondary antibodies (Dako EnVision + kit; Agilent, Santa Clara, CA, USA) were then applied for 30 min; and following further 20-min incubation at room temperature, the slides were colorized for 10 min in 3,3′-diaminobenzidine.

Bacterial suspension

A suspension of *Staphylococcusaureus* (1×10^8 CFU/mL) was prepared and collected by centrifugation. After washing (i.e., resuspension in sterile saline solution and centrifugation), the microbes were again suspended in sterile saline (1 mL), aliquoting 10 μL to each well of a 96-well plate (1×10^6 CFU/mL) for subsequent experimentation.

Selective precipitation of HSP27 or 70 in adenoidal lavage fluid

Adenoidal lavage fluid (1 mL) was precleared by incubation with protein G-Sepharose beads (Sigma-Aldrich) for 1 h at 4 °C. Precleared specimens were then incubated overnight with antibodies to HSP27 or 70, thereafter removing antibody-bound HSP by 2-h protein G-Sepharose precipitation at 4 °C. HSP recovery was confirmed by western blot analysis. Using the residual fluid (cleared of HSP27 or 70), we transferred 100 µL to each well of prepared bacterial plates individually seeded with *S.aureus* (1×10^6) in sterile saline (10 µL). The samples were then subjected to shaking incubation for 18 h at 37 °C, collected for serial dilution in saline solution, and eventually pour-plated onto Brain Heart Infusion agar. Colonies appearing on plates after 8 h of incubation at 37 °C were counted, and the population of *S.aureus* was calculated.

Statistical analysis

All data were expressed as mean ± standard deviation. Pearson's correlation coefficient was applied in comparing two continuous variables. Univariate and multivariate linear regression models served to analyze relations between HSPs and various demographic or clinical variables. *P* values < 0.05 were considered statistically significant in computations driven by standard software (SPSS v18; IBM Corp, Armonk, NY, USA).

Results

HSP27 and 70 expression levels in adenoidal tissues and lavage fluids of children

To determine if HSP27 or 70 is expressed in adenoidal tissue or in surface secretions, we collected 40 tissue specimens and lavage fluid samples (Table 1) from pediatric subjects for western blot analysis. HSP27 and 70 proved

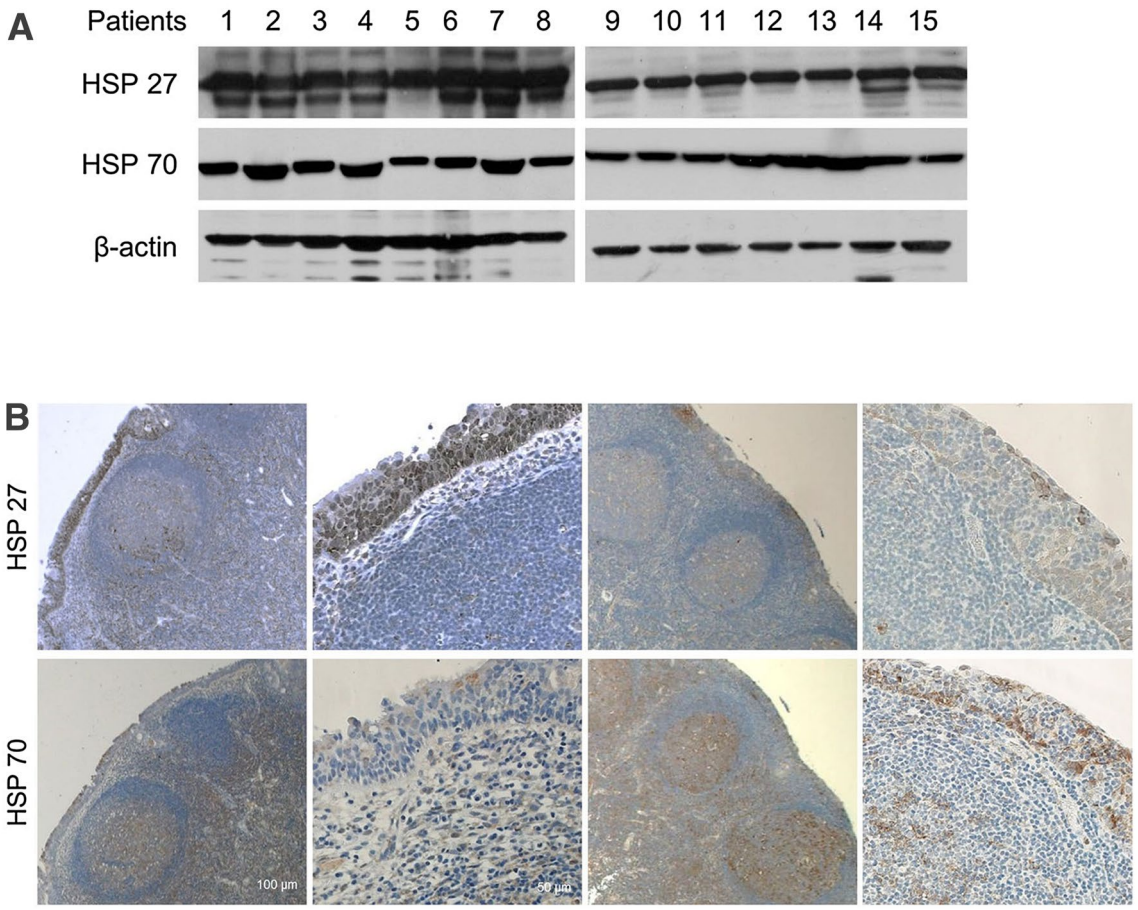

Fig. 1 **a** Adenoidal lysates analyzed via western blot for HSP27 and 70 expression levels (β-actin as loading control); and **b** immunohistochemical analysis of HSP27 and 70 in adenoidal tissues

Table 2 Quantification of HSP27, HSP70, and TNF-α in lavage of adenoidal secretions

	Value
HSP27	2564.82 ± 2631.78 (77.07–7130.18)
HSP70	2204.39 ± 2119.67 (139.16–8405.63)
TNF-α	
Positive samples	10 (25)
Expression level	269.68 ± 241.46 (37.35–604.37)

Data expressed as pg/mL ± SD (range) or *N* (%)

HSP heat shock protein, *TNF-α* tumor necrosis factor alpha, *SD* standard deviation

to be constitutively expressed in all adenoidal lysates (Fig. 1a). In immunohistochemical staining of processed tissue, these HSPs were largely confined to the cytoplasm of cells, present in epithelium, lymphoid follicles, and interfollicular areas. The epithelium was strongly positive for HSP27, whereas subepithelial regions showed strong HSP70 positivity (Fig. 1b). Washings of adenoidal secretions, analyzed by ELISA, regularly contained both HSP27 (mean 2564.82 ± 2631.78 pg/mL) and HSP70 (mean 2204.39 ± 2119.67 pg/mL). As a general inflammatory mediator, TNF-α (mean 269.68 ± 241.46 pg/mL) was detected in just 25% (10/40) of lavage samples (Table 2).

Adenoidal size and HSP70 expression level only show significant association

In evaluating factors related to the expression levels of HSP27 and 70, univariate and multivariate regression models were applied. Ultimately, we identified a significant association between HSP70 levels in adenoidal lavage fluid and adenoidal size, although this was not true of HSP27 (Table 3). Our analysis also demonstrated a significant relation between HSP27 and 70 that was inverse in nature (Fig. 2).

Tentative role of HSP27 and 70 in adenoidal immune response

Finally, we conducted antibacterial assays to examine the potential immunologic functions of HSP27 and 70 in adenoids of pediatric subjects. As a prominent bacterial inhabitant of upper airway mucosa, *S.aureus* was selected for this purpose. Treatment with HSP27 or 70 increased *S.aureus* bacterial numbers in a dose-dependent manner. The *S.aureus* population of controls (1×10^7 CFU/mL) rose to ~ 2.5×10^7 CFU/mL and to 1.8×10^7 CFU/mL after HSP27 and 70 treatment (10 ng/mL each), respectively (Fig. 3a). Furthermore, the growth of *S.aureus* was partially inhibited by precipitation of HSP27 or 70 from adenoidal lavage fluids (Fig. 3b).

Table 3 Univariate and multivariate linear regression analyses of factors implicated in HSP27 or HSP70 expression

Variables	Univariate		Multivariate	
	Standardized coefficients	*P* value	Standardized coefficients	*P* value
HSP27				
Age	−0.199	0.275	−0.056	0.831
BMI	−0.104	0.577	0.193	0.360
Allergic rhinitis	−0.059	0.728	0.248	0.202
WBC count	0.196	0.298	0.045	0.811
Eosinophil count	0.388	0.050	0.363	0.060
Size of adenoids	−0.247	0.14	−0.020	0.932
HSP 70	−0.398	0.015	−0.536	0.047
HSP70				
Age	0.248	0.171	0.366	0.079
BMI	0.139	0.457	−0.001	0.997
Allergic rhinitis	0.005	0.978	0.248	0.124
WBC count	0.009	0.964	0.111	0.479
Eosinophil count	−0.105	0.608	0.003	0.984
Size of adenoids	0.731	< 0.001	0.515	0.003
HSP27	−0.398	0.015	−0.376	0.047

HSP heat shock protein, *BMI* body mass index, *WBC* white blood cell

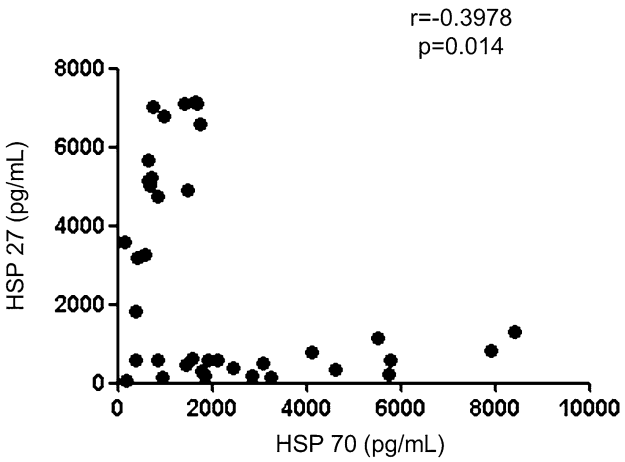

Fig. 2 Negatively correlating HSP27 and 70 levels in statistical analysis of adenoidal lavage fluid from children. *r* Pearson's correlation coefficient

Discussion

HSP27 and 70 are major cytoprotective chaperones [14]. They act to restore damaged proteins and ensure their delivery to appropriate intracellular sites, thus conferring

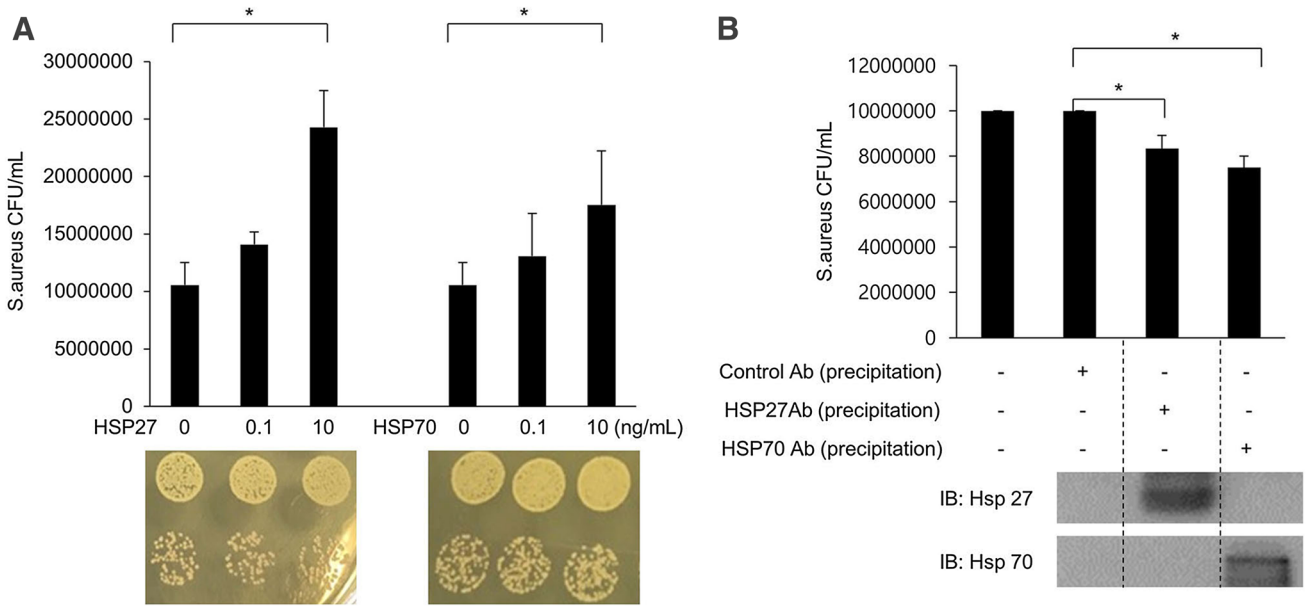

Fig. 3 HSP27 and 70 increase counts of *Staphylococcus aureus*: **a** HSP27 or 70 at concentration shown applied to each well of 96-well plate containing 1×10^6 CFU/mL of *S.aureus* for further 16-h incubation. Samples then serially diluted (saline solution) and pour-plated onto Brain Heart Infusion agar, counting colonies after 8-h incubation at 37 °C (bottom) and calculating bacterial population; **b** HSP27 or 70 precipitated from adenoidal lavage fluids using anti-HSP 27 or anti-HSP 70 (bottom). Lavage fluid (depleted of HSP27 or 70) applied to wells containing 10 µL of sterile saline with 1×10^6 CFU/mL of *S. aureus,* then placed in shaking incubator at 37 °C for 18 h. Collected samples serially diluted (saline solution) and pour-plated onto Brain Heart Infusion agar, counting colonies after 8-h incubation at 37 °C and calculating bacterial population. ($N=3$, $P<0.05$ significant)

protection [15]. Recently, the anti-inflammatory properties of HSP27 and 70 have also been demonstrated. Exogenous HSP27 is known to increase IL-10 expression by human monocytes, and in instances of inflammatory bowel disease or arthritis, IL-10 production is induced by HSP70 [16, 17]. Such findings indicate that HSP27 and 70 serve as immunomodulatory agents in various organs [18–20]. However, their presence and function in the adenoids of children are rarely studied.

An earlier immunohistochemical study has documented broad expression of HSP70 throughout the adenoids, including mucosal epithelium, germinal centers, interfollicular lymphocytes, and vascular endothelium [4]. However, its simplified analysis was confined to adenoidal tissues only (i.e., no extracellular testing) and was not inclusive of other HSPs. To confirm the immunologic status of HSPs as DAMPs, evidence of their extracellular presence is critical. Hence, the dual focus of this present investigation, showing that HSP27 and 70 are indeed regularly present within adenoidal tissues and lavage fluids alike. We also sought to evaluate clinical factors that may impact levels of HSPs and implicate immunologic functions. To our knowledge, this is the first reported study of HSP27 and 70 expression levels in both tissue and secretions of adenoids from pediatric subjects.

In another past study, adenoidal enlargement was shown to affect antimicrobial peptide expression levels [21]. Consequently, we anticipated that adenoidal size may reflect extracellular HSP levels. Although this was true of HSP70 in adenoidal lavage fluids, no such relation was evident for HSP27. Results of our immunostaining also revealed differing distributions of HSP27 and 70 within the adenoids. (Fig. 1b), and we found that assayed levels of HSP27 and 70 negatively correlated. It thus appears that the expression and secretion of HSP27 and 70 may be differentially regulated in the adenoids. Because there is no published data as yet in this regard, we have launched in vitro experiments to further explore this premise.

Of note, the fact that TNF-α was identified in only 25% (10/40) of the present cohort is aligned with the outcomes of a previous study we conducted, showing detectable TNF-α in 14 of 42 nasopharyngeal secretion samples collected from children [22]. These data overall suggest that HSP27 and 70 may be more important regulators of regional immunity than are systemic inflammatory cytokines.

Certain researchers have indicated that secreted antimicrobial peptides and cytokines may be important regulators of ion channels, angiogenesis, and wound repair in the airways [23]. To address the immunologic roles of HSP27 and 70, we used *S.aureus* (a common upper airway pathogen) in performing preliminary antibacterial assays. Both HSP27 and 70 separately increased bacterial numbers in vitro; and upon the precipitation of each from adenoidal lavage fluids,

bacterial numbers declined. These are tentative findings, however, affording no conclusive immunologic link. Still, such observations cannot be discounted and may implicate both HSPs as immune mediators in the adenoids. A large population-based microbial analysis in this setting, aimed at HSP27 and 70 expression levels, is, therefore, warranted.

The present study has several limitations, particularly the lack of adenoidal epithelial cells in primary culture and the undocumented origins of secreted HSPs. We are currently at work on a culture system to remedy these deficits. Although our rudimentary antibacterial assays proved inconclusive, a large and more sophisticated population-based study may also help clarify the immunologic ramifications of HSP27 and 70 alluded to herein.

In conclusion, this is the first known study to examine adenoidal expression levels of HSP27 and 70. Through our efforts, we identified a significant association between levels of HSP70 and adenoidal size, and the negatively correlating aspects of these two HSPs emerged. Our preliminary experimentation also suggests that HSP27 and 70 may participate in immune responses by regulating bacterial proliferation.

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Author contributions HJM wrote the manuscript, JSP performed experiments. CEK was responsible for data collection, and KSK reviewed whole manuscript.

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Compliance with ethical statements

Conflict of interest The authors have no conflicts of interest to declare.

Ethical approval This study was approved by the Institutional Review Board of Chung-Ang University College of Medicine (C2015092[1550]).

Informed consent Informed consent was obtained from all of our enrolled subjects.

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