Article title: Hydrogel-based delivery of Tat-fused protein Hsp70 protects dopaminergic cells *in vitro* and in a mouse model of Parkinson's disease

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SUPPLEMENTARY INFORMATION S2 (Acrobat file)

Text summary: Supplementary Material S2 reports additional results related to the characterization of Hsp70 and Tat-Hsp70, COLL-HA hydrogels, gelatin particles and Tat-Hsp70 release (*in vitro*).

1. Biochemical characterization of Hsp70 and Tat-Hsp70

Fig.1 shows the results of biochemical characterization of Hsp70 and Tat-Hsp70 by SDS-PAGE and circular dichroism.

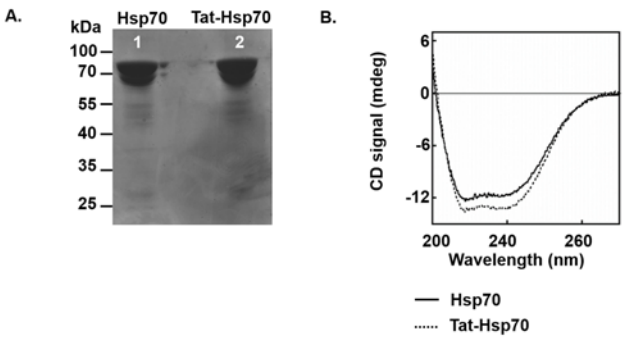

Figure 1: Purification and characterization of Hsp70 and Tat-Hsp70

A) SDS-PAGE analysis of recombinant Hsp70 (lane 1) and Tat-Hsp70 (lane 2) purified on a HiTrap Chelating column. We loaded 10 µg recombinant protein in each lane. B) Comparison of the far-UV circular dichroism spectra of 0.1 mg/mL Hsp70 (solid line) and Tat-Hsp70 (dotted line). We recorded both spectra at 15°C in 10 mM Tris-HCl buffer, pH 8.0, 250 mM KCl.

2. Biological characterization of Hsp70 and Tat-Hsp70

Dose-response curves for SH-SY5Y cells exposed to 6-OHDA or H_2O_2

Fig.2 shows SH-SY5Y cell metabolic activity (measured by resazurin assay) and morphology (observed by phase-inverted optical microscopy) after incubation with 6-OHDA and H_2O_2 for 24h.

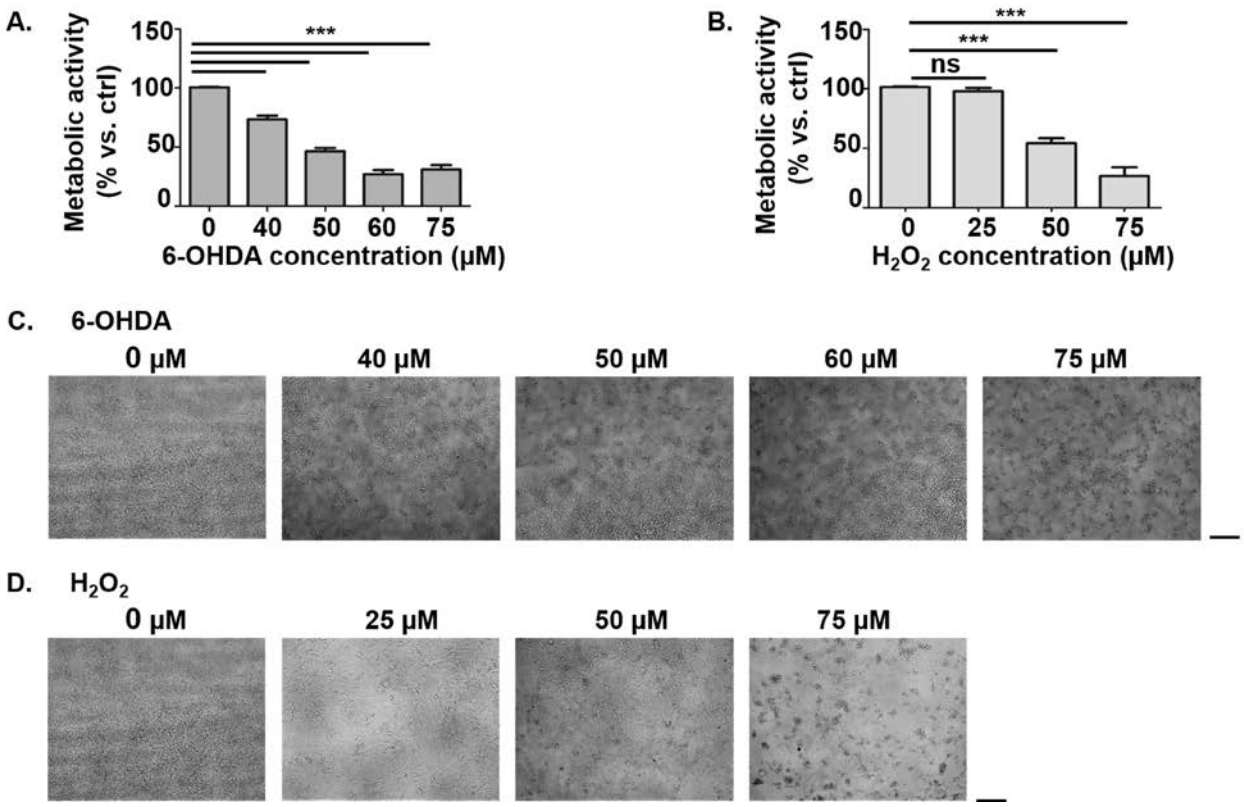

Figure 2: Dose-response curves for SH-SY5Y cells exposed to 6-OHDA or H_2O_2

SH-SY5Y cell metabolic activity after 24h incubation with: A) 6-OHDA (40, 50, 60, 75 µM). Results from resazurin assay, 13 replicates/group. Fifty µM 6-OHDA reduced cell metabolic activity by 53% (mean value). B) H_2O_2 (25, 50, 75 µM). Results from resazurin assay, 9 replicates/group. Fifty µM H_2O_2 reduced cell metabolic activity by 46% and 75 µM H_2O_2 by 73% (mean values).

Results are mean ± SD. We normalized the results to control (0 µM, left bar) in standard medium. We analyzed the results with one-way ANOVA followed by Tukey's multiple comparisons test. ns, $p>0.05$; ***, $p<0.001$.

3. Morphological characterization of the hydrogels

Fig.3 shows the results of ultrastructural analysis by scanning and transmission electron microscopy, the study of COLL-HA interactions by optical microscopy after staining with Picrosirius red and Alcian blue and the investigation of COLL bioactivity by immunofluorescence on COLL, COLL-LMW HA and COLL-HMW HA hydrogels.

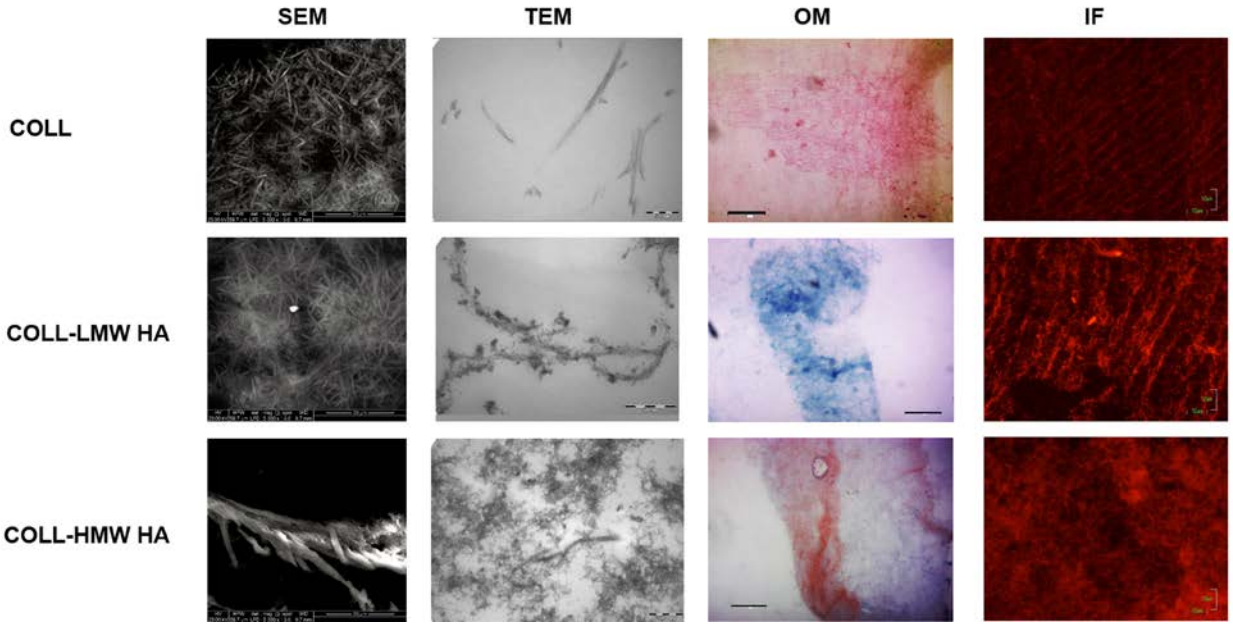

Figure 3: Morphological characterization of the hydrogels (3 replicates/group)

SEM images of COLL, COLL-LMW HA, COLL-HMW HA. Scale bar 20 µm.

TEM images of COLL, COLL-LMW HA, COLL-HMW HA. Scale bar 500 nm.

Optical microscopy (OM) images of hydrogel slices stained by Picrosirius red and Alcian blue. COLL is stained red, HA is stained blue. 100X magnification of COLL and COLL-LMW HA (scale bar 10 µm), 20X magnification of COLL-HMW HA (scale bar 50 µm).

Immunofluorescence (IF) images obtained by incubating hydrogel slices with anti-COLL type I antibody, followed by detection with Cy3-conjugated secondary antibody (red). COLL scale bar 100 µm; COLL-LMW HA and COLL-HMW HA: scale bar 10 µm.

SEM analysis showed that COLL assembles to create 3D fibrillar networks and its bundles have a wavy

course. TEM analysis clearly showed COLL fibrils and suggested that HA modifies the network structure by filling the spaces between COLL fibrils and coating their network.

After staining with Picrosirius red and Alcian blue (staining COLL red and HA blue, respectively), only red areas were visible in COLL gels, as expected. COLL-LMW HA gels were stained blue, with no evident red areas. The network appeared homogeneous, with HA covering COLL fibrils, as suggested by TEM analysis. On the contrary, COLL-HMW HA gels showed separated red or blue-stained areas. In addition, purple-stained areas were visible because of COLL and HA entanglements. These features indicated that the network of COLL-HMW HA gels is interconnected, but non-homogeneous.

Immunostaining with the anti-COLL I antibody suggested that COLL (stained red) partly keeps its bioactivity after the preparation of the semi-IPNs. In fact, a regular COLL fibril pattern was apparent in COLL and COLL-LMW HA, but not clearly visible in COLL-HMW HA gels, confirming the previous finding.

4. Particle characterization

4.1 *Particle morphology and size distribution*

We performed NTA at 25°C in water. A monochromatic laser beam at 488 nm illuminated the diluted samples ($1.66 \cdot 10^9 \pm 1.67 \cdot 10^8$ particles/mL; 5 replicates) to register a 60s video with a mean frame rate of 30 frames/s. We used the NTA software (version 3.0, NanoSight) to identify and track each particle on a frame-by-frame basis, then to track and measure its Brownian movement from frame to frame. We obtained particle hydrodynamic size from its velocity by applying the two-dimensional Stokes-Einstein equation.

Video 4 shows a sample video record (10 s) of moving gelatin particles registered during NTA. It shows that the majority of the particles has a spherical morphology. Because of limitations in file size, we uploaded a low quality video.

4.2 *Cell morphology after incubation with gelatin particles*

Fig.5A shows the morphology of SH-SY5Y cells (observed by phase-inverted optical microscopy and fluorescence microscopy) after incubation with gelatin particles (0, 25, 500 μg/mL) for 24h. For fluorescence images, cell nuclei are stained by DAPI, while F-actin in the cytoskeleton is stained with phalloidin.

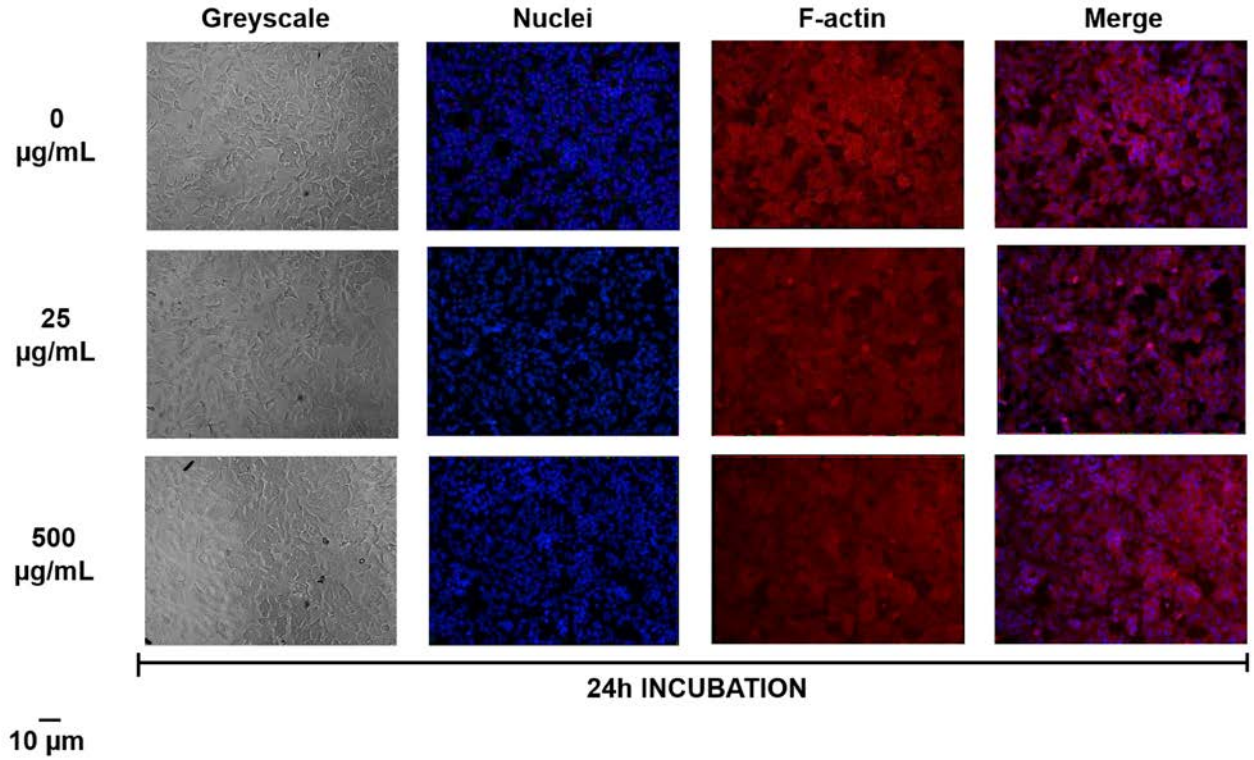

Figure 5A: Morphology of SH-SY5Y cells after incubation with gelatin particles for 24h

Phase-inverted optical microscopy and fluorescence microscopy showed the morphology of SH-SY5Y cells (4 replicates/group) after 24h incubation with gelatin particles (0, 25, 500 μg/mL). Cell nuclei are stained blue by DAPI, while F-actin in the cytoskeleton is stained red with phalloidin. As apparent, cells conserved their morphology, with no signs of nuclear or architectural degradation. Scale bar 10 μm.

Fig.5B shows the morphology of SH-SY5Y cells (observed by phase-inverted optical microscopy and fluorescence microscopy) after incubation with gelatin particles (0, 25, 500 µg/mL) for 72h. For fluorescence images, cell nuclei are stained by DAPI, while F-actin in the cytoskeleton is stained with phalloidin.

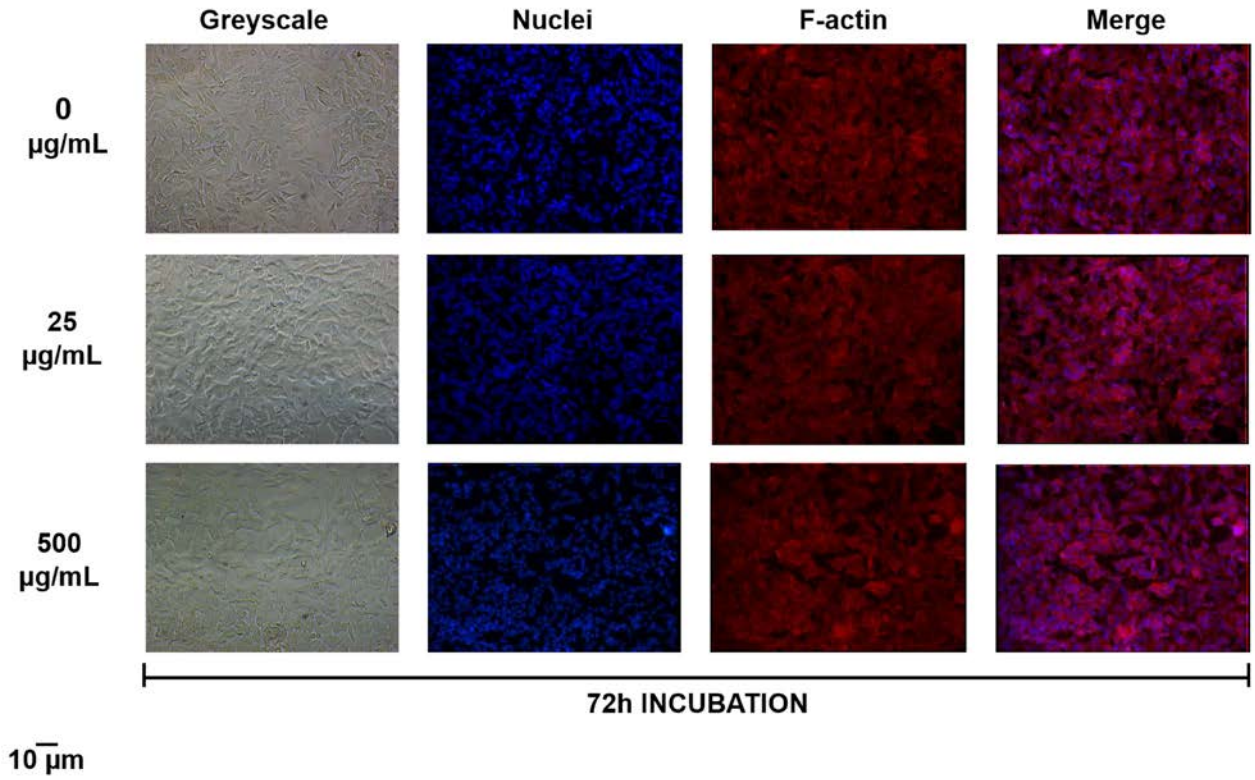

Figure 5B: Morphology of SH-SY5Y cells after incubation with gelatin particles for 72h

Phase-inverted optical microscopy and fluorescence microscopy showed the morphology of SH-SY5Y cells (4 replicates/group) after 72h incubation with gelatin particles (0, 25, 500 µg/mL). Cell nuclei are stained blue by DAPI, while F-actin in the cytoskeleton is stained red with phalloidin. As apparent, cells conserved their morphology, with no signs of nuclear or architectural degradation. Scale bar 10 µm.

5. Characterization of Tat-Hsp70-loaded composites

5.1 *Neuroprotective effect of Tat-Hsp70 released from COLL-LMW HA composites*

Fig.6 shows the neuroprotective effect of Tat-Hsp70 released from COLL-LMW HA composites after exposing SH-SY5Y cells to 6-OHDA (or H_2O_2). We observed neuroprotection when cell metabolic activity (measured by resazurin assay) increased with respect to incubation with 50 µM 6-OHDA (or H_2O_2) only.

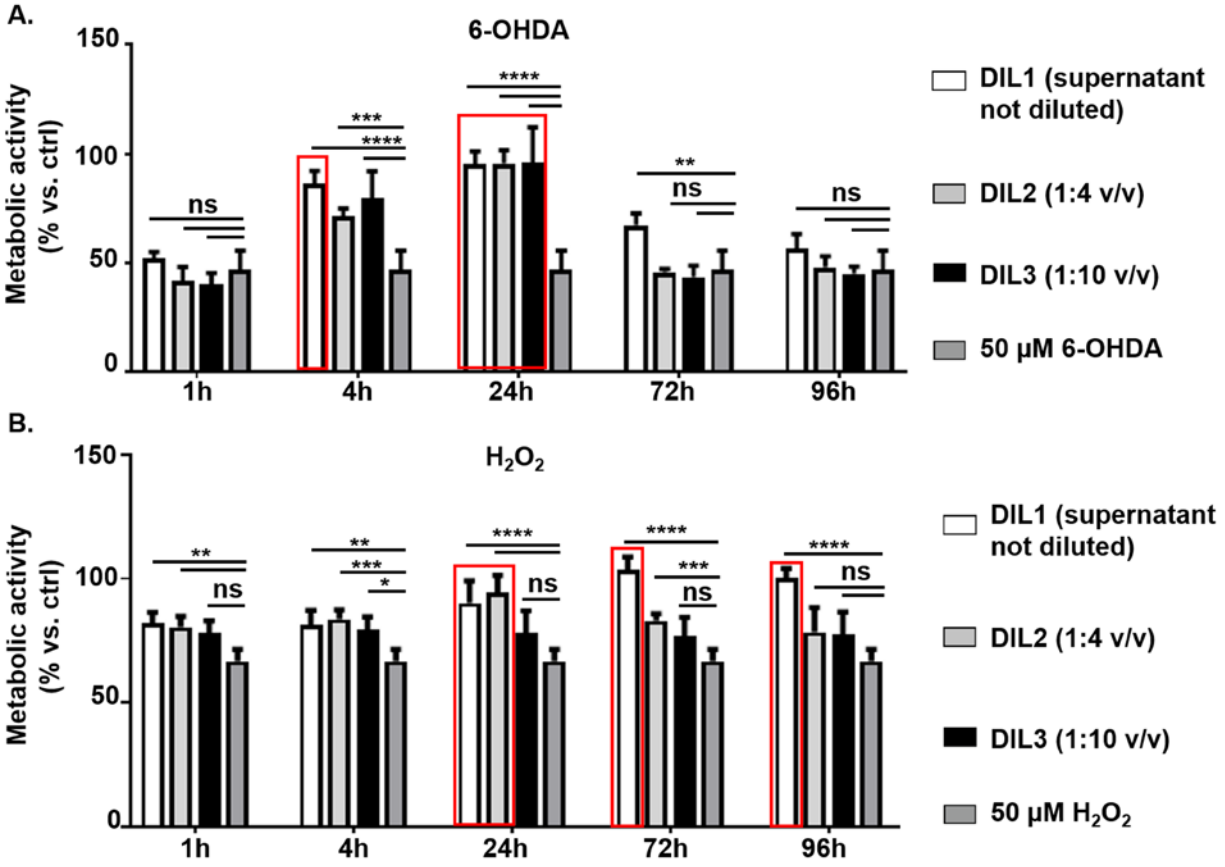

Figure 6: Neuroprotective effect of Tat-Hsp70 released from COLL-LMW HA composites

We incubated SH-SY5Y cells for 2h with supernatants collected from Tat-Hsp70-loaded composites after 1, 4, 24, 72 and 96h in culture medium, then for 24h with: A) 50 µM 6-OHDA; B) 50 µM H_2O_2. We transferred supernatants (undiluted or diluted with medium: DIL1, not diluted; DIL2, 1:4 v/v; DIL3, 1:10 v/v) to cells. Results (mean ± SD) from resazurin assay, 4 replicates/group. We analyzed the results with one-way ANOVA followed by Tukey's multiple comparisons test. ns, $p>0.05$; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. The red rectangles highlight the conditions in which supernatants increased cell metabolic activity to levels comparable with controls (not shown) (ns, $p>0.05$). In the same experiment, we also

confirmed the neuroprotective effect of free Tat-Hsp70 250 nM, in agreement with Fig.1A-B (data not shown).

Cell metabolic activity recovered in comparison to samples treated with 50 μM 6-OHDA (Fig.6A) after incubation with supernatants collected after 4h (all dilutions), 24h (all dilutions) and 72h (DIL1). Supernatants collected after 4h (DIL1) and 24h (all dilutions) raised cell metabolic activity to levels comparable with controls (ns, $p>0.05$).

For samples treated with 50 μM H_2O_2 (Fig.6B), cell metabolic activity recovered after incubation with supernatants collected after 1h (DIL1 and DIL2), 4h (all dilutions), 24h (DIL1 and DIL2), 72h (DIL1 and DIL2) and 96h (DIL1). Supernatants collected after 24h (DIL1 and DIL2), 72h and 96h (DIL1) raised cell metabolic activity to levels comparable with controls (ns, $p>0.05$).

5.2 *Neuroprotective effect of hydrogel degradation products*

After 168h (7 days) *in vitro* the selected composites were still macroscopically compact. However, we observed a softening of the upper part, suggesting the release of polymer components. For this reason, we evaluated the possible neuroprotective action of degradation products from unloaded COLL-LMW HA composites.

Fig.7 shows the results related to the presence of COLL in the supernatants from Tat-Hsp70-loaded composites, indicating hydrogel degradation. It also confirms the cytocompatibility of the supernatants from unloaded COLL-LMW HA composites and the absence of neuroprotective effects in the presence of both oxidative stimuli (6-OHDA or H_2O_2).

Silver staining confirmed the presence of COLL (Fig.7A), while we deduced the presence of HA by ultrastructural analysis (see Supplementary Material S2, Section 3, Fig.3), demonstrating that HA is not covalently bound to COLL, but fills the spaces between COLL fibrils and coats their network.

In the absence of toxic stimuli (Fig.7B), polymer components had no impact on SH-SY5Y cell metabolic activity, with results comparable to controls ($p>0.05$). In the presence of oxidative stimuli, they did not show

neuroprotection, with results comparable to 50 µM 6-OHDA or H_2O_2 (p>0.05, Fig.7C and 7D, respectively). These results confirmed that unloaded composites were ineffective against oxidative stressors.

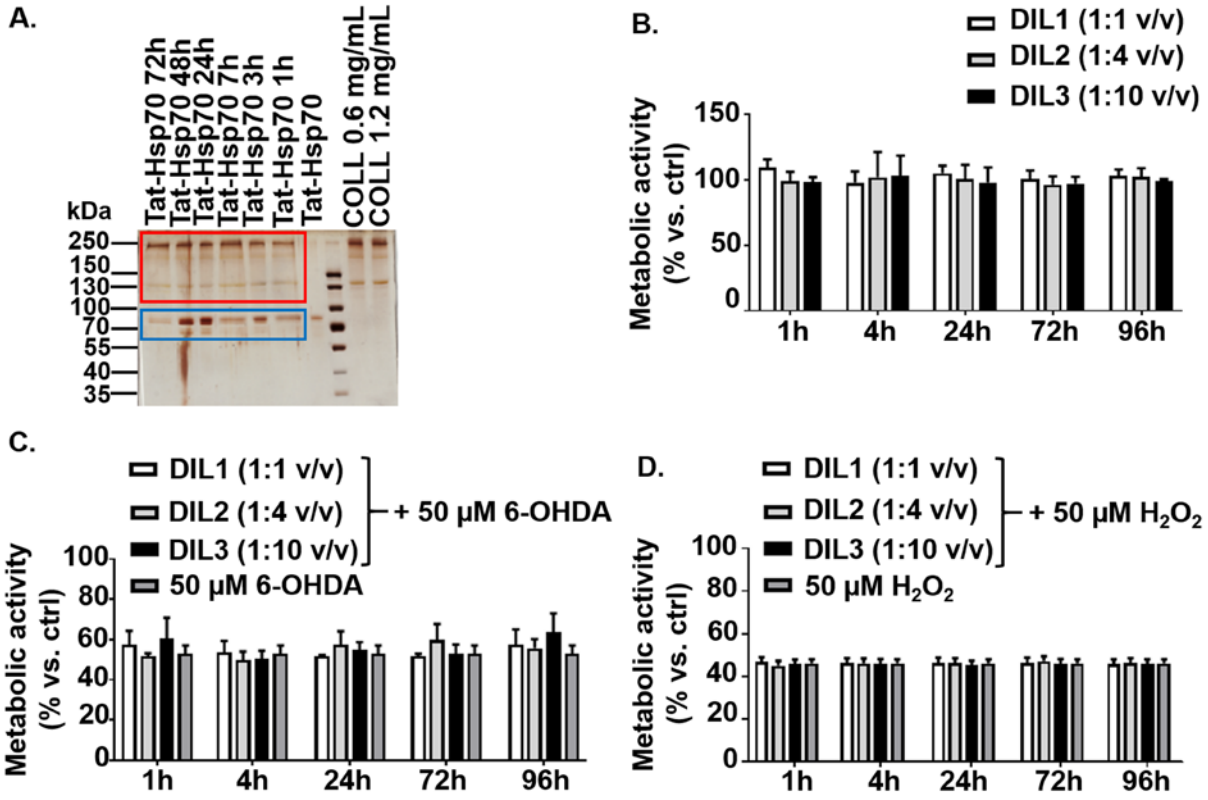

Figure 7: Degradation of Tat-Hsp70-loaded composites and effects of the supernatants from unloaded COLL-LMW HA composites on SH-SY5Y metabolic activity in the presence and absence of 6-OHDA and H_2O_2

A) After electrophoretic separation on 8-15% gradient polyacrylamide gels, we performed silver staining on the supernatants collected from Tat-Hsp70-loaded composites in PBS. From left to right: supernatants collected 72h (60 µL), 48h (20 µL), 24h (10 µL), 7h (60 µL), 3h (20 µL) and 1h (60 µL) after dipping in PBS, free Tat-Hsp70 (0.1 µg), COLL solution (0.96 µg) to a final concentration of 0.6 and 1.2 mg/mL. In the lanes loaded with supernatants, the red rectangle shows the bands corresponding to COLL, while the blue one shows the bands corresponding to Tat-Hsp70.

We incubated SH-SY5Y cells for 2h with supernatants collected from COLL-LMW HA composites after 1, 4, 24, 72 and 96h in culture medium, then for 24h with: B) culture medium; C) 50 µM 6-OHDA; D) 50 µM

H_2O_2. We transferred supernatants (undiluted or diluted with medium: DIL1, not diluted; DIL2, 1:4 v/v; DIL3, 1:10 v/v) to cells. Results (mean ± SD) from resazurin assay, 4 replicates/group. We analyzed the results with one-way ANOVA followed by Tukey's multiple comparisons test. $p>0.05$ for all comparisons.