Comparative in vivo experiments: investigation of cortex tissue

Education, Learning

This comparative in vivo experiment used 28 male Wistar rat pups (Rattus norvegicus) purchased from LASCO Biotechnology at 8 weeks of age. The animals were maintained in plastic cages with sawdust bedding and fed a standard diet with filtered water access provided ad libitum. They followed a 12-hour light-dark cycle with a controlled ambient temperature and were acclimatized for 1 month before the procedure began. All applicable rules concerning ethical treatment of animals were followed using the guidelines laid out by the Taiwan Society for Laboratory Animals Sciences for the care and use of laboratory animals.

ADSC experimental procedure

ADSCs were isolated from the adipose tissue of the 3 month old rats following a previously described method (Ren et al. 2012). The primary ADSCs were then cultured in low glucose Dulbecco's Modified Eagle's Medium (DMEM-D2902; Sigma-Aldrich, St. Louis, MO, USA) with 10% Fetal Bovine Serum and 1% penicillin/streptomycin up to 80% confluence before subculturing with a 0. 25% trypsin/0. 02 mM EDTA solution, and seeding at a density of 1 x 104 cells/cm2. The rats were divided into five groups. The control group (N= 7) did not receive any treatment, the ADSC group (N= 6) received ADSCs, the ADSC + EGCG group (N= 6) received ADSCs co-cultured with 10μM of EGCG, the ADSC + miR-3575 mimic group (N= 4) received ADSCs co-cultured with 10nM of miR-3575, and the ADSC + miR-3575 inhibitor group (N= 5) received ADSCs co-cultured with 20nM of miR-3575 inhibitor. All treated rats received 1 x 106 ADSCs at 20 months of age and were sacrificed at 22 months for analyses.

Tissue extraction

The whole brains of all rats were extracted, blood was removed with ice cold PBS, the brain tissues were kept in 4% Paraformaldehyde, and the cortex tissue was separated from the hippocampus with extra attention towards the prefrontal cortex. The tissue was homogenized in lysis buffer (iNtRON Pro Prep, cat. 17081) at a concentration of 100mg tissue/3ml of buffer and then placed in -80℃ for 12 hours. The homogenates were then placed on ice for 15 minutes and then centrifuged at 12, 000 rpm for 40 minutes. The supernatant from each sample was collected and stored in -80℃ for future analyses.

Hematoxylin-Eosin Staining

The cortex tissues were first placed in formalin then encased in paraffin. The slides were immersed in a series of ethanol concentrations (100, 95 and 75%), each for 15 minutes, then stained with hematoxylin and eosin (H&E), and finally rinsed with water. The slides were then dehydrated through serial ethanol concentrations for 15 minutes each, cleaned with xylene, and had their coverslips placed on. Photomicrographs were taken using a Zeiss Axiophot microscope (Zeiss, Oberkochen, Germany). The photos taken all concentrate on the same area of prefrontal cortex at 200x magnification to better observe the cell morphology of the tissues.

Protein Quantification

A Bradford protein assay was performed using protein dye (Bio-Rad, Richmond, CA, USA, cat. 500-0006) and Bovine Serum Albumin (UniRegion Bio-Tech, UR-BSA-001). BSA was first used as the protein standard in serial

dilutions with double deionized water (ddH2O), to which the protein assay dye was added, into a 96-well plate and incubated at room temperature for 5 minutes. Changes in optical density were then measured at 595 nm and plotted to elicit the standard curve. Quantification of the cortex samples was done in triplicate by adding previously collected supernatant to the protein dye in a 96 well plate, measuring the absorbance at 595 nm, and comparing the results against the standard curve formula in order to determine accurate protein concentrations. The samples were diluted with ddH2O and loading dye as required and placed on a 100℃ hot plate for 10 minutes then an ice bath for 10 minutes before being stored in -20℃ while not in use.

Western Blot analysis

SDS-PAGE was performed with 8-13. 5% polyacrylamide gels (Table 1) to denature and separate the proteins before transferring them to a 0. 45µm polyvinylidene difluoride membrane (PVDF) (Millipore, USA, IPVH00010) and then blocking the membrane with 5% milk, (Anchor, New Zealand) prepared with 100 mM Tris-HCl, 0. 9% NaCl, and 0. 1% (vol/vol) Tween 20, adjusted to pH 7. 4 (TBST), for one hour incubation at room temperature to prevent non-specific binding. The membranes were then incubated at 4℃ overnight with the relevant primary antibodies (Table 2) in TBST at a 1: 2000 dilution. The membranes were washed in TBST three times, 10 minutes each, and were then incubated with the appropriate horseradish peroxidase conjugated secondary antibodies (Santa Cruz Biotech, sc-2004, sc-2005, sc-2020) for 1 hour at a 1: 4000 dilution. After washing the membranes as before, they were placed in a Chemiluminescent HRP substrate (Millipore, Darmstadt,

Germany, WBKLS0500) and the immunoblotted proteins were quantified using a Fujifilm LAS-4000 imaging system (Fujifilm, Tokyo, Japan). All quantitative analyses were done with ImageJ (NIH, MD, USA) and Excel (Microsoft, USA) with the significance assessed by Student’s T-test where a p-value < 0. 05 is considered statistically significant.

Results

H&E Stains show moderate neuroregeneration with ADSC and ADSC + Mimic treatments and a marked improvement with ADSC + EGCG. The H&E Stains (Fig. 1a) revealed distinct differences among the overall integrity of the neural cells. In the aged control group, the striations of degenerative empty space are easily noticeable and linear. Injection of ADSCs improved the integrity of the tissue, with many of the smaller pockets filled in and the results were mirrored in the ADSC + Mimic group with a similar degree of restoration, however the EGCG group showed the best result with nearly all empty space filled in and a proliferation of nuclei relative to ADSC. Quantitative analysis verified these results (Fig. 1b), but also showed that the ADSC + Mimic group did very similarly to the ADSC group alone. The inhibitor group, however, indicated a lack of major improvement due to many small remaining pockets as shown on the photomicrograph.

Recognized miRNA-3575 targets exhibit no change on Western Blotting.

Repeated western blotting results have shown inconclusive evidence for the influence of miRNA-3575 on the recognized protein targets, Hif-1???? and FoxO3a (Fig. 7). In addition, similar to the H&E stain quantifications, no

significant improvement was noted in any of the proteins tested beyond what was seen with the ADSC group.

ADSC + EGCG strongly upregulated proteins related to survival and longevity.

Sirt-1 and the proteins on the same signaling pathway, PGC-1α and p-AMPKα, were upregulated in tandem when ADSC + EGCG was administered compared to ADSC alone, confirming previous studies in which EGCG increased detectable levels of SIRT-1 and p-AMPKα (Fig. 4). Similarly, levels of p-AKTs473 (Fig. 2) were up regulated the most with the EGCG group, but an inverse outcome was seen with P53 western blots with the EGCG group's result coming out lower than the control or ADSC. Surprisingly, ADSC + Inhibitor showed a similarly low P53 result (Fig. 5).

ADSC + EGCG increased both endogenous gluthathione and HO-1.

HO-1 and Nrf-2 (Fig. 3) were not upregulated with the administration of ADSCs but showed a substantial increase when combined with EGCG, while P-ERK was also slightly induced. ADSCs were likely better able to promote the endogenous anti-oxidant, glutathione, in the aged rat cortex as confirmed with the increased levels of the upstream signaling protein and indicator, Cystathionine gamma-lyase (CTH).

ADSC + EGCG increased production of BDNF, signaling neuroregeneration.

The neurotrophic factor, BDNF (Fig. 6), was strongly upregulated with the administration of ADSC + EGCG which indicates an environment conducive

to neural growth and survival. The increase was seen in Pro-BDNF levels and was stronger than the ADSC treatment alone. To verify this trend, TrkB, a neural receptor for BDNF also increased in tandem with the EGCG group, while active BDNF decreased in an inverse correlation.

Discussion

EGCG is an important and promising polyphenol with mechanisms that can positively affect the entire body. However, normal methods of consuming EGCG, most commonly through drinking green tea, rarely give us high enough doses of EGCG to provide a strong neural effect, as the concentration that crosses the blood brain barrier is only about 2. 8% per 30 minutes of what is consumed (Pervin et al. 2017). In this study, we were looking for novel methods of introducing this catechin into an aged rat model by co-culturing young ADSCs from its own body with EGCG and determining whether the treatment was more effective than ADSCa alone. The results showed that many of the benefits of either ADSCa or EGCG were enhanced when both were combined and injected intravenously. It’s possible that the ADSCs were able to permeate the blood brain barrier more effectively than EGCG could unaided, and once inside the cortex, factors from the EGCG may have been secreted out as the ADSCs proliferated. In addition, the EGCG likely made the ADSCs more robust via increased cell proliferation signaling and increased mitochondrial biogenesis.

Visually, the H&E stain photomicrographs of the ADSC + EGCG group showed a marked improvement in the integrity of the aged cortex tissue where the control was plagued with lesions of empty space where the brain had shrunk.

This is likely due to the EGCG creating an environment of increased neuron survival and inhibiting Nogo-A, a powerful myelin derived neurite outgrowth inhibitor (Gundimeda et al. 2014). An increase of Pro-BDNF and Tyrosine Kinase B (TrkB), a BDNF receptor, contributed to this supportive environment of neuronal growth as both of them were significantly upregulated as confirmed by western blot statistics (Figure 6). The tests also showed that active BDNF decreased sharply hinting to an uptake of the nerve growth factor by the budding cells. At the same time, the ADSCs were not only able to help repair the tissue, but triggered neighboring neurons to do the same, as it’s been shown that stem cell secreted factors also have a rejuvenating effect on tissues (Zhao et al. 2017).