

COMPOSITIONS AND METHODS FOR TREATING STROKE

FIELD OF THE INVENTION

The specification relates generally to the field of compositions and methods for treating stroke.

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BACKGROUND OF THE INVENTION

Stroke is the second leading cause of death and third leading cause of disability and death worldwide and results in significant economic and societal cost. A stroke results in a complex interplay of inflammation and repair with effects on neural, vascular, and connective tissue in and around the affected areas of the brain. Unfortunately, treatment options for stroke are still greatly limited. Intravenous recombinant tissue plasminogen activator (tPA) and endovascular thrombectomy (EVT) are currently the only effective treatments available for acute stroke. However, there is only a brief window of opportunity where they can be successfully applied. There are also no treatments that offer restoration of function and as a result, many patients are left with residual deficits following a stroke. Cell-based therapies have shown promising results in animal models addressing the recovery phase following stroke (Savitz et al. 2004). This is encouraging as currently, there are no approved treatment options addressing the reversal of neurological damages once a stroke has occurred.

There is thus an urgent need for adjunctive therapies for stroke such as neuroprotective agents and cell therapy.

SUMMARY OF THE INVENTION

The present inventors have found that amnion cell therapy can be used to treat patients with stroke or symptoms of stroke. For example, amnion cell therapy can be used for treatment of post-stroke inflammation, including inflammation in brain tissue.

Accordingly, in one aspect the present invention provides a method of treating stroke and/or symptoms of stroke in a subject in need thereof comprising administering human amniotic epithelial cells (hAECs) in an amount in the range of 2–8 x 10⁶/kg to the subject.

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In another aspect, the present invention provides a method of treating stroke and/or symptoms of stroke in a subject in need thereof comprising administering human amniotic epithelial cells (hAECs) in an amount in the range of about 100–700 x 10⁶ cells to the subject.

In one embodiment, the administration may be by any suitable route, for example, parenterally, for example, by intramuscular, subcutaneous, or intravenous or intra-arterial injection.

In one embodiment, the administration is by intravenous transfusion.

5 In one embodiment, the dose of hAECs is about 2×10^6 cells/kg.

In one embodiment, the dose of hAECs is about 4×10^6 cells/kg.

In one embodiment, the dose of hAECs is about 8×10^6 cells/kg.

In one embodiment, the dose of hAECs is an amount in the range of about 100×10^6 to about 700×10^6 cells.

10 In one embodiment, the dose of hAECs is an amount in the range of about 160×10^6 to about 230×10^6 cells.

In some embodiments, the dose of hAECs is about 320×10^6 cells.

In some embodiments, the hAECs are given as one dose.

In one embodiment, the one dose is given over a period of about 1 hour.

15 In one embodiment, the total dose of hAECs is divided into sub-doses and administered at two or more separate time intervals.

In one embodiment, the divided total dose is administered at dosage intervals over two or more subsequent days following the first dose.

20 In one embodiment, the total dose is about 4×10^6 /kg hAECs administered intravenously in sub-doses over two consecutive days.

In one embodiment, the total dose is about 320×10^6 hAECs administered intravenously in sub-doses over two consecutive days.

In one embodiment, the dose is administered in two split doses of about 160×10^6 hAECs administered intravenously in sub-doses over two consecutive days.

25 In one embodiment, the hAECs are administered in combination with another treatment or treatment regime.

In one embodiment, the total dose or the first sub-dose of hAECs is administered to a subject from between 0 to 24 and a second dose is administered to a subject from between 24 to 48 hours post-stroke.

30 In one embodiment, the total dose or the first sub-dose of hAECs is administered to a subject from immediately post stroke up to seven days post-stroke.

In one embodiment, the total dose or the first sub-dose of hAECs is administered to a subject immediately from post stroke up to 48 hours post-stroke.

35 In one embodiment, the total dose or the first sub-dose of hAECs is administered to a subject immediately from post stroke up to one day, two days, three days, four days, five days, six days, or seven days post-stroke.

In one embodiment, the total dose or the first sub-dose of hAECs is administered to a subject immediately from post stroke up to 28 days post-stroke.

In one embodiment, the total dose or the first sub-dose of hAECs is administered to a subject from immediately post stroke up to three days post-stroke.

5 In some embodiments, hAECs are administered any time from immediately post-stroke to any time while inflammation persists, for example, in one or several doses.

In one embodiment, the total dose or a first sub-dose of hAECs is administered to a subject whilst post-stroke inflammation is present. In one
10 embodiment, the inflammation is in the brain tissue of a subject.

In some embodiments, the hAECs are administered in a cryopreserved composition.

In one embodiment, the cryopreserved composition comprises sterile saline containing 2% human serum albumin (HSA) and 0.16% dimethyl sulphoxide (DMSO)
15 at a ratio of 3:1.

In one embodiment, the cryopreserved composition comprises sterile saline containing 2% HSA and 0.32% DMSO at a ratio of 3:1.

In one embodiment, hAECs are positive for EpCAM and negative for mesenchymal stromal markers CD90 and CD105.

20 In one embodiment, EpCAM+CD90-CD105- cells are further gated for stem cell and stromal markers.

In one embodiment, hAECs express embryonic stem cell surface markers.

In one embodiment, the embryonic stem cell surface markers comprise one or more or all of: stage-specific embryonic antigen-3 (SSEA-3); stage-specific embryonic
25 antigen-4 (SSEA-4); octamer-binding transcription factor-4 (OCT-4); tumour rejection antigen1-60 (TRA1-60); and tumour rejection antigen 1-80 (TRA1-80).

In one aspect, provided herein is the use of hAECs in the manufacture of a medicament for treatment of stroke or symptoms of stroke in a subject in need thereof, wherein the hAECs are to be administered at a dose in the range of about 100×10^6
30 cells to about 700×10^6 cells.

In one embodiment, the medicament is administered up to three days post-stroke.

In one aspect, provided herein is a pharmaceutical composition comprising about $2-8 \times 10^6$ /kg hAECs for the treatment of stroke and symptoms of stroke in a
35 subject in need thereof, wherein the composition is administered up to three days post-stroke.

In one aspect, provided herein is a pharmaceutical composition comprising about 100×10^6 to about 700×10^6 hAECs for the treatment of stroke and symptoms of stroke in a subject in need thereof, wherein the composition is administered up to three days post-stroke.

5 Any embodiment herein shall be taken to apply *mutatis mutandis* to any other embodiment unless specifically stated otherwise. For instance, as the skilled person would understand examples of inhibitors and health conditions outlined above for the methods of the invention equally apply to the use and pharmaceutical compositions of the invention.

10 The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purpose of exemplification only. Functionally-equivalent products, compositions and methods are clearly within the scope of the invention, as described herein.

15 Throughout this specification, unless specifically stated otherwise or the context requires otherwise, reference to a single step, composition of matter, group of steps or group of compositions of matter shall be taken to encompass one and a plurality (*i.e.* one or more) of those steps, compositions of matter, groups of steps or group of compositions of matter.

20 The invention is hereinafter described by way of the following non-limiting Examples and with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE ACCOMPANYING DRAWINGS

25 **Figure 1: Action of amnion stem cells on the inflammatory cascade post ischaemic stroke.** Schematic depiction of injury pathways after stroke, and of the potential effects of hAECs on post-stroke inflammation and immunosuppression.

Figure 2: Schematic map of dose escalation and de-escalation approach. Map of the dose escalation and de-escalation stages performed similar to the classic 3 + 3 scheme in a Phase 1 trial whereby the sample size is increased in small increments of 3. For a 5-dose escalation, the estimated sample size is 15. The Bayesian Optimal Interval Design (BOIN) (Liu and Yuan 2015) used here weighs the dose escalation by the lower boundary of the target toxicity rate (safety rate = $0.6 \times$ target) and de-escalation by the upper boundary (toxicity rate = $1.4 \times$ target). This method recommends setting the default target rate at 0.3 rather than a lower value because of the small sample size typical for a Phase 1 trial (Liu and Yuan 2015).

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Figure 3: Timeline showing the animal models used. (A) Young male mice (7-14 weeks) were subjected to transient middle cerebral artery occlusion (tMCAO) and received either vehicle or hAECs (10^6) at either 1.5 h (acute) or 3 d (delayed) post-stroke. Functional outcome was assessed at the timepoints indicated, and at the end of the experiment organs were harvested for analysis. (B) Young male mice (7-14 weeks) were subjected to permanent middle cerebral artery occlusion (pMCAO) and received either vehicle or hAECs (10^6) at 1.5 h post-stroke. Functional outcome was assessed at approximately 23 h post-stroke, and at the end of the experiment brains were harvested for analysis. (C) Cohort 1: Aged female mice (20-22 months) were subjected to photothrombotic stroke (PT) of the primary motor cortex and received either vehicle or hAECs (10^6) at 1-14 d post-stroke. Functional outcome was assessed over 56 d, and at the end of the experiment brains were removed for analysis. Cohort 2: Aged female mice were subjected to photothrombotic stroke and received either vehicle or hAECs (10^6) at 1 d post-stroke. At the end of the experiment, brains and lymphoid organs were removed for analysis. (D) Adult marmosets (3-5 years; male and female) were subjected to endothelin-1 (ET-1) -induced ischemia of the primary visual cortex and received either vehicle or hAECs (5×10^6) at 1.5 h post-stroke. Magnetic resonance imaging was performed to measure infarct volume at 1 d and 10 d post-stroke.

Figure 4: Characterisation of human amnion epithelial cells (hAECs). (A) Primary hAEC isolates were gated positive for epithelial cell marker EpCAM and negative for mesenchymal stromal markers CD90 and CD105. (B) Cells that were EpCAM+CD90-CD105- were further gated for the stem cell and stromal markers CD146, STRO-1, CD44, SSEA-4, CD29 and CD73.

Figure 5: Human amnion epithelial cells (hAECs) reduce cerebral apoptosis in vivo but provide limited direct protection against neuronal cell death *in vitro*. *In vivo*: Young male mice were subjected to 30 min MCAO and were injected with either vehicle or hAECs 1.5 h post-stroke. (A) Immunohistochemistry was used to quantify annexin V-positive cells within the infarct core and peri-infarct region at 72 h post-stroke (n=7 per group). Western blotting was used to quantify whole brain protein expression of: (B) cleaved-caspase 3 (n=2-4 per group) as well as (C) total and cleaved caspase-8 (n=3-4 per group). *In vitro*: primary neuronal cultures were exposed to either normal conditions (NC) or glucose deprivation (GD) for 24 h. (D) Glucose-deprived cultures were treated with either vehicle or hAECs (104 or 105 cells per dish) and dead cells were quantified (n=5 per group). *P<0.05, **P<0.01, two-way ANOVA (A), Student's

unpaired t-test (C) or one-way ANOVA followed by Tukey's multiple comparison test (D). Data are presented as mean $\pm$ SEM.

- Figure 6: Human amnion epithelial cells (hAECs) reduce post-stroke inflammation.** Young male mice were subjected to either sham surgery or 30 min MCAO and were injected with either vehicle or hAECs 1.5 h later. **(A)** Flow cytometry was performed to quantify total leukocytes, monocytes/macrophages, neutrophils, B cells, CD4+ T cells and CD8+ T cells in the right/ischemic brain hemisphere of sham, vehicle- and hAECs-treated mice at 24 h post-surgery (n=4-13 per group). **(B)** Western blot analysis of nod-like receptor protein 3 (NLRP3) expression in the brain of hAECs- and vehicle- treated mice at 24 and 72 h post-stroke (n=3-4 per group). *P<0.05, **P<0.01, ***P<0.001, one-way ANOVA, followed by Newman-Keuls multiple comparison test (A) or Student's unpaired t-test (B). Data are presented as mean $\pm$ SEM.
- Figure 7: Acute administration of hAECs blunts hallmarks of stroke-induced systemic immunosuppression.** Young male mice were subjected to either sham surgery or 60 min MCAO and were injected with either vehicle or hAECs 1.5 h later. At 24 or 72 h post-surgery spleens were removed for analysis. **(A)** Number of immunoreactive cleaved-caspase 3+ (cl. casp 3+) splenocytes at 24 and 72 h post-stroke (n=3-5). **(B)** Spleen weights at 24 and 72 h post-stroke (n=9-10). **(C)** Flow cytometric analysis of splenic: total leukocytes, monocytes, neutrophils, B cells, CD4+ T cells and CD8+ T cells at 72 h post-stroke (n=6-13). *P<0.05, **P<0.01, ***P<0.001, one-way ANOVA followed by Tukey's multiple comparison test. Data are presented as mean $\pm$ SEM. Keuls multiple comparison test (A) or Student's unpaired t-test (B). Data are presented as mean $\pm$ SEM.

- Figure 8: Young male mice were subjected to either sham surgery or 60 min MCAO and were injected with either vehicle or hAECs 1.5 h later.** At 72 h post-surgery blood was withdrawn for analysis by flow cytometry. Numbers of circulating: total leukocytes, monocytes, neutrophils, B cells, CD4+ T cells and CD8+ T cells (n=9-13). *P<0.05, **P<0.01, ***P<0.001, one-way ANOVA followed by Tukey's multiple comparison test. Data are presented as mean $\pm$ SEM.

Figure 9: Human amnion epithelial cells (hAECs) reduce infarct volume and the expression of splenic T cell transcription factors but have no effect on post-stroke lung infections following stroke. Young male mice were subjected to either sham surgery or 60 min MCAO and were injected with either vehicle or hAECs 1.5 h later. **(A)** mRNA expression of Th1 transcription factors Tbx21 and Stat4, Th2 transcription factors Gata3 and Stat6, Th17 transcription factor Rorc and T regulatory cell transcription factor Foxp3 within the spleen of sham, vehicle- and hAECs-treated mice at 72 h post-surgery (n=9-10) per group. **(B)** Lungs were collected for bacteriological analysis at 72 h post-surgery. Data are expressed as colony forming units (CFU)/mg lung tissue (Log10) (n=7-12 per group). **(C)** Infarct volume measured at 24 and 72 h following 60 min MCAO (n=8-10 per group). *P<0.05, ***P<0.001, one-way ANOVA, followed by Tukey's multiple comparison test (A), Newman Keuls multiple comparison test (B) or Student's unpaired t-test (C). Data are presented as mean $\pm$ SEM.

Figure 10: Delayed administration of human amnion epithelial cells (hAECs) improves functional recovery following stroke. Young male mice were administered either hAECs or vehicle at 3 d post-30 min MCAO and functional recovery was assessed over 14 d. **(A)** latency to fall in hanging grip test, **(B)** latency to remove stickers on forepaws and **(C)**, survival of hAECs- and vehicle-treated mice over 3 to 14 d post-stroke (n=6-17). *P<0.05, **P<0.01, two-way ANOVA followed by Bonferroni's test or Log-rank test (for survival).

Figure 11: Human amnion epithelial cells (hAECs) do not alter infarct size in aged female mice at 56 d post photothrombotic stroke, but increase glial scar density. **(A)** Lesion volume at 56 d post-stroke (n=7-8 per group). **(B)** Glial scar area and density at 56 d in mice treated with either hAECs or vehicle at 1 d post-stroke (n=6-7 per group). *P<0.05, one-way ANOVA (A) or Student's unpaired t-test (B). Data are presented as mean $\pm$ SEM.

Figure 12: Human amnion epithelial cells (hAECs) prevent the decrease in inhibitory interneurons within the peri-infarct cortex of aged-female mice following photothrombotic stroke. Immunofluorescence was performed to quantify the amount of glutamic acid decarboxylase-67 (GAD67) staining in the peri-infarct cortex in the brains of sham-, stroke + vehicle- and stroke + 1 d hAEC-treated animals at 7 d post-surgery (n=6 per group; data are expressed as mean fluorescence

intensity). * $P < 0.05$, *** $P < 0.001$ (one-way ANOVA followed by Tukey's multiple comparison test). Data are presented as mean $\pm$ SEM.

5 **Figure 13: Human amnion epithelial cells (hAECs) modulate the systemic immune response to photothrombotic stroke in aged female mice.** Flow cytometry was used to quantify the percentage of: **(A)** myeloid CD11b+ cells (n= 5-8 per group), **(B)** B220+ B cells (n=3-9 per group) and **(C)**, CD3+ T cells (n=4-8 per group) within the blood or bone marrow, spleen and lymph node at 7 d after stroke. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA followed by Tukey's multiple
10 comparison test. Data are presented as mean $\pm$ SEM.

Figure 14: Administration of mouse embryonic stem cells (mESCs) has no effect on post-stroke outcomes. Young male mice were subjected to either sham surgery or 30 min MCAO and were injected with either vehicle or mESCs. Functional outcome
15 and/or infarct volume was assessed at 24 post-stroke. **(A)** Latency to fall on hanging grip test (n=4 per group). **(B)** Median neurological deficit score (n=4 per group). **(C)** Infarct volumes measured using thionin staining (n=4 per group). Data are presented as $\pm$ SEM.

20 **Figure 15: Acute administration of human amnion epithelial cells (hAECs) reduces functional deficits and infarct volume after stroke.** Young male mice were subjected to either sham surgery or 30 minute transient middle cerebral artery occlusion and injected with vehicle or hAECs 1.5 hours later. Functional outcome and infarct volume were assessed at 24, 48, and 72 hours postsurgery. **(A)** Latency to fall
25 on hanging grip test (n=7–19). **(B)** Latency to remove stickers on forepaws (n=5–11). **(C)** Neurological deficit score (n=7–19). **(D)** From top to bottom, Infarct volumes measured using thionin, representative images of magnetic resonance scans delineating infarct area at 24, 48, and 72 hours, infarct volumes measured by magnetic resonance imaging (MRI) at 24, 48, and 72 hours compared with 72 hours thionin
30 (n=7–12). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, 1-way ANOVA and Tukey test (A and B), Kruskal–Wallis test and Dunn test (C), or Student unpaired t test (D). Data are mean $\pm$ SEM (except neurological deficit scores presented as median).

Figure 16: Intravenously administered human amnion epithelial cells (hAECs) migrate to the brain in a CXCR4-dependent manner after stroke and elicit neuroprotection in intact but not splenectomized (SX) mice. (A) Representative images of (B) and (C). (B) Young male mice were subjected to either sham surgery or 30 minute transient middle cerebral artery occlusion (MCAO), and carboxyfluorescein succinimidyl ester (CFSE)-labeled hAECs were injected 1.5 hours later. CFSE-labeled hAECs were counted in brain or spleen at 24 and 72 hours (n=2–8). (C) Young male mice were subjected to 30 minute transient MCAO and injected with CFSE-labelled hAECs and vehicle or CXCR4 antagonist, AMD3100. From left to right, CFSE-labelled hAECs per brain section, latency to fall on hanging grip test, and neurological deficit scores (n=5). (D) Young male SX mice were subjected to 60 minute transient MCAO and injected with either vehicle or hAECs at 1.5 hours. From left to right, Infarct volume and latency to fall on hanging grip test assessed at 24 hours poststroke (n=7). Scale bar indicates 100 μ m. *P<0.05; **P<0.01, Student unpaired t test. Data are mean $\pm$ SEM (except neurological deficit scores, presented as median).

Figure 17: Acute administration of human amnion epithelial cells (hAECs) reduces cerebral apoptosis and inflammation after stroke. Young male mice were subjected to either sham surgery or 30 minute middle cerebral artery occlusion and injected with vehicle or hAECs 1.5 hours later. Apoptotic and immune cells were quantified in the brain at 24 and 72 hours post surgery. (A) Representative images showing co-localisation of neuronal marker NeuN and cleaved caspase-3, and immunohistochemical analysis of immunoreactive cleaved caspase-3+ (cl. casp 3+) cells within the infarct core and peri-infarct region (n=6–9). Scale bars represent 100 μ m. Immunohistochemistry was used to quantify: (B) myeloperoxidase (MPO)+ cells (n=3–7), (C) CD3+ cells (n=4–7), and (D) F4/80+ cells (n=3–5) and 3-nitrotyrosine (3NT)+ F4/80+ cells (n=5) per ischemic/right hemisphere section. *P<0.05; **P<0.01; ***P<0.001, 2-way ANOVA (A), 1-way ANOVA followed by Newman–Keuls test (B–D), or Student unpaired t test (D). Data are mean $\pm$ SEM.

Figure 18: Efficacy of human amnion epithelial cells (hAECs) after permanent cerebral ischemia. Young male mice were subjected to permanent middle cerebral artery occlusion and injected with vehicle or hAECs at 1.5 hours. Functional outcome, infarct volumes, and brain immune cell numbers were assessed at 24 hours. (A) From left to right, Latency to remove adhesive labels from forepaws, latency to fall in hanging grip test, and median neurological deficit score (n=7–12). (B) Infarct volume

(vehicle: n=9; hAECs: n=12). **(C)** Immunohistochemical quantification of infiltrating CD3+ T cells per ischemic section (n=7/group). **(D)** Immunohistochemical quantification of infiltrating myeloperoxidase (MPO)–positive neutrophils/field of view (n=7). Arrows indicate positive cells, scale bars represent 50 μ m. *P<0.05, Student unpaired t test (A–D) except for neurological deficits scores which were compared using a Mann–Whitney test. Data are presented as mean $\pm$ SEM except neurological deficit scores, presented as median.

Figure 19: Administration of human amnion epithelial cells (hAECs) improves long-term outcome after stroke in aged female mice and marmosets. (A) Mice were administered vehicle or hAECs at 1 or 14 days after sham surgery, or photothrombotic stroke and functional outcome were assessed for 56 days from left to right, Cylinder test assessing forepaw asymmetry, grid-walking test assessing forepaw and hindpaw deficits (n=7–8). ***P<0.001 (stroke vs sham), +P<0.05 (stroke+1 day hAECs vs stroke+vehicle), ^P<0.05 (stroke+1 and 14 days hAECs vs stroke+vehicle), ^^P<0.01 (stroke+1 and 14 days hAECs vs stroke+vehicle), 2-way ANOVA followed by Tukey' test. **(B)** Mice were administered vehicle or hAECs 1 day postphotothrombosis and infarct volume and histological markers of peri-infarct plasticity were assessed at 7 days from top to bottom, left to right: Infarct volume at 7 days (n=6), immunohistochemical analysis of 4',6-diamidino-2-phenylindole (DAPI), MAP2 (microtubule-associated protein-2), and GFAP (glial fibrillary acidic protein) of sham, vehicle- and hAECs-treated animals (n=6), representative images are shown above. Yellow boxes in the representative vehicle image show the regions of interest that were subjected to analysis. *P<0.05; **P<0.01; ***P<0.001, Student unpaired t test. **(C)** Adult marmosets were subjected to endothelin-1–induced ischemia and injected with saline- or hAECs at 1.5 hours. Magnetic resonance (MR) imaging assessed infarct volumes at day 1 and day 10. From left to right, Representative MR scans delineating infarct area, mean infarct volume, and change in infarct volume from day 1 to day 10 in saline- and hAECs-treated marmosets (n=3; 2 males and 1 female/group). No statistical comparisons were made. All data are presented as mean $\pm$ SEM.

Figure 20: Functional outcome and infarct volume of mice at 24 h post-stroke. Mice subjected to 1 h transient middle cerebral artery occlusion and tPA-treated at the time of reperfusion were treated with vehicle or hAECs at 1.5 h post-stroke. Functional outcome was evaluated by **(A)** clinical score (n=14-23 per group), **(B)** latency to fall on hanging grip test (n=9-11 per group), and **(C)** infarct volume (n=9-11 per group). Data

are presented as mean $\pm$ SEM, except for clinical score presented as median. (A) Clinical score was compared using a Kruskal-Wallis test. (B and C) One-way ANOVA test with Dunnett's post hoc test.

- 5 **Figure 21: Clinical score of sham mice at 24 h following vehicle or tPA+vehicle treatment.** Data are presented as median and compared using a Mann-Whitney test (n=7–8 per group).

- 10 **Figure 22: Infarct distribution and volume. (A)** Comparison of infarct distribution based on bregma location (n = 6–11 per group). Infarct size at **(B)** 3 h (n = 7 per group) and **(C)** 6 h (n = 10–11 per group) post-stroke. Representative images of delineated infarcts at 6 h are shown in **(D)**. Data are presented as mean $\pm$ SEM, *p < 0.05 compared with tPA + vehicle, (b) one-way ANOVA test with Dunnett's post hoc test, (c) Kruskal-Wallis test with Dunn's *post hoc* test.

- 15 **Figure 23: Human amnion epithelial cells reach the ipsilateral hemisphere at 6 h post-stroke. (A)** Immunofluorescence staining for human HLA-G (green, arrows) revealed the presence of hAECs in the ischemic hemisphere. Nuclei are identified by DAPI counterstain (blue). **(B)** Number of HLA-G+ cells in the ipsilateral hemisphere (n = 9 mice; average of 6 sections per mouse). Objective: 40X.
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- Figure 24: Representative images of intracerebral haemorrhage in the ipsilateral hemisphere at 6 h post-stroke. A–C** Arrows indicate haemorrhages. **D–E.** Correlation between IgG staining and infarct volume in all mice (D), or between IgG intensity and HLA-G+ (hAEC) cells in tPA+hAEC-treated mice (E). Coefficient of determination (R²) and p values are indicated in each graph.
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- Figure 25: Blood-brain barrier disruption at 6 h post-stroke. (A)** Representative images of brain sections showing IgG leakage (anti-mouse antibody) into the ipsilateral hemisphere after stroke. **(B)** Quantification of extravasation of IgG in terms of intensity (n=9-11 per group). **(C)** Number of MMP-9+ cells in the ipsilateral hemisphere of the brain (n=6-11 per group). Data are expressed as mean $\pm$ SEM. One-way ANOVA test with Dunnett's post hoc test. Scale bar = 200 μ m. Objective: 10X.
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Figure 26: Immune cell numbers in the ipsilateral hemisphere at 6 h post-stroke. Quantification of **(A)** microglia/macrophages (Iba-1+ cells, n = 5–7 per group), **(B)** neutrophils (MPO+ cells, n = 8–10 per group), **(C)** macrophages (F4/80+ cells, n = 9–11 per group), and **(D)** 3-nitrotyrosine (3NT)+ F4/80+ cells (n = 9–11 per group) in the ipsilateral hemisphere at 6 h post-stroke. **(A, C, D)** were evaluated by immunofluorescence, whilst **(B)** was assessed by immunohistochemistry. Data are expressed as mean $\pm$ SEM. **(A)** Kruskal-Wallis test with Dunn's *post hoc* test. **(B-D)** One-way ANOVA test with Dunnett's *post hoc* test.

Figure 27: Representative images (A) microglia/macrophages (Iba-1+ cells), **(B)** neutrophils (MPO+ cells), **(C)** macrophages (F4/80+ cells), nuclei are identified by DAPI counterstain (blue) and **(D)** 3-nitrotyrosine (3NT)+ F4/80+ cells in the ipsilateral hemisphere at 6 h post-stroke. Objectives: 40X for A, C, D and 20X for B.

Figure 28: Administration of human amnion epithelial cells (hAECs) improves long-term outcome after stroke in 15-month-old mice. Mice were administered intravenous vehicle or hAECs at 7 days after photothrombotic stroke in the left primary motor cortex and functional outcome (right forepaw impairment that causes left-sided forepaw preference) was assessed over 56 days using the cylinder test for forepaw asymmetry. $^{**}P < 0.05$ (vehicle vs hAECs), 2-way ANOVA followed by Tukey' test. Vehicle-treated mice = circles (n=9 mice); hAECs-treated mice = squares (n=10 mice).

Figure 29: Infarct volume and FLAIR-DWI mismatch. The post-treatment was on the day that the hAEC infusion was completed. For patients receiving a dose of 8×10^6 cells/kg the MRI was on day 2. The unit of hAEC dose was 10^6 cells/kg. FLAIR, fluid attenuated inversion recovery sequence; DWI, diffusion weighted imaging.

Figure 30: Haematological profile. No particular pattern was observed among the eight patients. The unit of hAEC dose was $\times 10^6$ cells/kg.

Figure 31: Lymphocyte subset over time. No particular pattern was observed among the eight patients. The unit of hAEC dose was $\times 10^6$ cells/kg.

Figure 32: Inflammatory markers. No particular pattern was observed among the eight patients. The unit of hAEC dose was $\times 10^6$ cells/kg.

DETAILED DESCRIPTION OF THE INVENTION**General Techniques and Definitions**

Unless specifically defined otherwise, all technical and scientific terms used herein shall be taken to have the same meaning as commonly understood by one of
5 ordinary skill in the art (e.g., in cell culture, cell biology, regenerative medicine, stroke biology and treatment thereof, neurology, pharmacology and biochemistry).

Unless otherwise indicated, the cell culture and immunological techniques utilised in the present invention are standard procedures, well known to those skilled in the art. Such techniques are described and explained throughout the literature in
10 sources such as, J. Perbal, A Practical Guide to Molecular Cloning, John Wiley and Sons (1984), J. Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory Press (1989), T.A. Brown (editor), Essential Molecular Biology: A Practical Approach, Volumes 1 and 2, IRL Press (1991), D.M. Glover and B.D. Hames (editors), DNA Cloning: A Practical Approach, Volumes 1-4, IRL Press (1995 and 1996),
15 and F.M. Ausubel *et al.* (editors), Current Protocols in Molecular Biology, Greene Pub. Associates and Wiley-Interscience (1988, including all updates until present).

As used herein, the term about, unless stated to the contrary or otherwise defined in the context of the specification, refers to +/- 10%, more preferably +/- 5%, of the designated value.

20 Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

As used in this application, the term "or" is intended to mean an inclusive "or" rather than an exclusive "or". That is, unless specified otherwise, or clear from context, "X employs A or B" is intended to mean any of the natural inclusive permutations. That is, if X employs A; X employs B; or X employs both A and B, then "X employs A or B" is satisfied under any of the foregoing instances. Further, at least one of A and B and/or the like generally means A or B or both A and B. In addition, the articles "a" and "an" as
25 used in this application and the appended claims may generally be construed to mean "one or more" unless specified otherwise or clear from context to be directed to a singular form.

The terms "treating" or "treatment" as used herein, refer to both direct treatment of a subject by a medical professional (e.g., by administering a therapeutic agent to the
35 subject), or indirect treatment, effected, by at least one party, (e.g., a medical doctor, a nurse, a pharmacist, or a pharmaceutical sales representative) by providing instructions,

in any form, that (i) instruct a subject to self-treat according to a claimed method (e.g., self-administer a drug) or (ii) instruct a third party to treat a subject according to a claimed method. Also encompassed within the meaning of the term “treating” or “treatment” are prevention or reduction of the disease to be treated, e.g., by
5 administering a therapeutic at a sufficiently early phase of disease to prevent or slow its progression.

The term “at risk” as used herein, refers to a probability of developing a health condition that is higher than in the general population. Accordingly, treatment of an individual considered to be “at risk” of a particular condition is designed to prevent a
10 subject from the developing the condition or at least to reduce the risk of developing the condition to a level no higher than that found in general population as a whole.

The terms “co-administration” or the like, as used herein, are meant to encompass administration of the selected therapeutic agents to a single patient, and are intended to include treatment regimens in which the agents are administered by the
15 same or different route of administration or at the same or different time.

Stroke

Stroke refers to a group of conditions caused by an interruption of blood supply to the brain, leading to brain tissue damage. The consequences of stroke include motor,
20 sensory, visual, and neuropsychological symptom deficits. Stroke is broadly categorised into two main types: haemorrhagic and ischaemic. Haemorrhagic stroke is caused by a bursting of blood vessels and bleeding in the brain, and accounts for approximately 13% of stroke patients. Ischaemic stroke is due to an interruption in blood supply such as a blockage or clot, and accounts for approximately 87% of stroke patients (Gupta and
25 Sharma 2023). Both haemorrhagic and ischaemic stroke have further subclassifications, of which the skilled person will be aware. For example, widely accepted classification systems for ischemic stroke subtypes based upon either phenotypic or causative nature includes Trial of Org 10,172 in Acute Stroke Treatment (TOAST).

Treatment of stroke

Therapeutic options for stroke include recombinant tissue plasminogen activator (tPA), the only FDA-approved anti-thrombotic drug. tPA is a powerful blood thinner, and its use comes with risks of serious bleeding-related complications and side-effects. A further limitation of tPA is that administration must occur within a time window
35 of 4.5 hours after onset of stroke. This means that a patient must access care and

haemorrhagic stroke must be excluded before they can receive tPA. tPA remains one of the best stroke treatments available for those who can use it.

The inventors of the present application have identified a treatment for stroke that does not induce bleeding and which is effective in treating stroke up to 7 days post-stroke. Without being bound by theory, the inventors of the present application have identified a treatment for the inflammatory aspects of stroke. The treatment does not induce bleeding and is therefore suitable for treatment of both haemorrhagic and ischaemic stroke, and can be given immediately, before any blood-thinner suitability is determined. The treatment is therefore also effective as long as inflammation persists.

Human amnion epithelial cells

The term “human amnion epithelial cell (hAEC)” as used herein, refers to cells derived from the innermost layer of the amniotic sac that forms part of the term placenta closest to the foetus and in direct contact with the amniotic fluid (amniotic membrane). These cells are also commonly referred to as human amniotic epithelial cells. hAECs are obtained from readily available discarded term placentae, and so their use avoids invasive extraction procedures and significant ethical constraints. The isolation and qualification of hAEC as multipotent stem cells have been initially described more than 15 years ago, followed by a growing amount of evidence in support of their use in allogeneic (or xenogeneic) settings with no tumorigenic or rejection risks. hAECs are non-immunogenic, non-tumourigenic anti-inflammatory cells which can develop into cells of all three germ line lineages (ectoderm, mesoderm and endoderm), can produce osteogenic, adipogenic, and chondrogenic lineages under appropriate culture conditions, and can be used as seed cells for allogeneic cell therapies including tissue regeneration.

hAECs are also well-reported to migrate to injured or damaged tissue, and to differentiate into the appropriate cell type to facilitate repair of the injured or damaged tissue (see, for example, Zhang and Lai 2020).

As described herein, hAECs are immunologically inert with low levels of human leukocyte antigens A, B, C, and DR—key antigens involved in transplant rejection—as well as expression and release of HLA-G, which is able to directly suppress immune responses. Thus, hAEC transplantation does not require adjunct immunosuppressant administration. Human amnion cells have been extensively characterised and qualified, primarily for their stem characteristics and functional properties. hAECs lack telomerase enzyme and so do not form tumours, nor do they differentiate into fibroblasts. The

present inventors have reviewed their suitability as an inherently safe and amenable stroke therapy (Broughton et al. 2013; Evans et al. 2018).

As described herein, isolated hAECs express surface markers of embryonic stem cells such as stage-specific embryonic antigen-3 and 4 (SSEA-3 and SSEA-4),
5 octamer-binding transcription factor-4 (OCT-4), and tumor rejection antigen 1-60 and 1-80 (TRA1-60 and TRA1-80). The expression of these markers does not exclude expression of additional markers, as will be apparent to the skilled person. hAECs isolated at term preserve the expression of different critical surface molecules, such as stage-specific embryonic antigens (SSEA), initially described on human embryonic stem
10 cells (ESC) and commonly detected on every pluripotent stem cell (iPS included). Both SSEA-3 and SSEA-4 are constitutively expressed on every hAEC batch, at different levels according to gestational age, but negative for SSEA-1 (a murine isoform) (Morandi et al 2023).

In addition, hAEC express keratan sulfate-associated antigens, such as tumour rejection antigens (TRA) 1-60 and TRA1-81, and preserve such markers upon
15 enzymatic digestion. Characteristically, amnion epithelial cells are negative for surface enzymes and molecules commonly used to identify stromal cells (i.e. CD44, CD90, and CD105). Additionally, hAEC batch qualification analysis includes the absence of any other tissue markers, such as lack of expression for cell-specific markers such as CD31
20 (endothelial), CD45 (leucocytes), and Glycophorin A (erythrocyte).

Transcription factors such as homeobox protein Nanog and octamerbinding (Oct)-4 have been reported in addition to surface markers. hAECs have been differentiated into cell types of all three germ layers, namely endoderm (liver, pancreas), mesoderm (cardiomyocyte) and ectoderm (neural cells). Of note, hAEC have been
25 reported to preserve a stable and normal karyotype, in combination with the lack of the expression of telomerase, a key regulator of proliferative potential. hAEC are not tumorigenic in preclinical models or in human subjects.

Although hAECs possess stem cell-like characteristics, they do not exhibit unlimited proliferation and have proven limited proliferative capacity *in vitro*, using
30 standard culture conditions due to the lack of telomerase activity and there is therefore also no risk of tumorigenesis *in vivo*.

In one embodiment, hAECs are positive for EpCAM and negative for mesenchymal stromal markers CD90 and CD105. In one embodiment, EpCAM+CD90-CD105- cells are further gated for stem cell and stromal markers.

In one embodiment, hAECs express embryonic stem cell surface markers, for example, one or more or all of stage-specific embryonic antigen-3 and 4 (SSEA-3 and SSEA-4), octamer-binding transcription factor-4 (OCT-4), and tumor rejection antigen1-60 and 1-80 (TRA1-60 and TRA1-80).

5 To safely and effectively use hAECs to repair the function of injured tissues and treat diseases, it is essential that they are of high quality. Techniques for hAEC culture are well-known in the art. Cultured cells require dissociation from treated growth surfaces. Suitable methods of cell dissociation will be apparent to the skilled person. Briefly, there are three primary methods for tissue dissociation and frequently the
10 methods are combined. These include: enzymatic dissociation, chemical dissociation and mechanical dissociation. Enzymatic dissociation is the process of using enzymes to digest cut-up tissue pieces thereby releasing cells from tissue. One suitable method of obtaining viable hAECs is enzymatic dissociation using the trypsin/EDTA technique. The key aspects involved in the isolation of hAECs with high yield, viability, and purity
15 are known in the art, see, for example, Motedayyen et al 2017. Standard manufacturing and cryopreservation that result in isolation of highly purified hAECs with reproducible and high viability in accordance with current good manufacturing procedure (GMP) requirements are also known in the art, see for example Gramignoli et al 2016. Serum-free protocols are also known in the art, see for example, Yang et al 2018, which
20 have obvious benefit for clinical applications.

Cryopreservation

Cryopreservation is the use of very low temperatures to preserve structurally intact living cells and tissues. Unprotected freezing is normally lethal, and the
25 mechanisms involved to produce stable cooling conditions that preserve life are well-known in the art. Briefly, the biological effects of cooling are dominated by the freezing of water, which results in the concentration of the solutes that are dissolved in the remaining liquid phase. Theories behind the mechanism of damage caused by freezing include that either ice crystals pierce or tease apart the cells, destroying them by direct
30 mechanical action, or that damage is from secondary effects via changes in the composition of the liquid phase. Cryoprotectants, simply by increasing the total concentration of all solutes in the system, reduce the amount of ice formed at any given temperature. To be biologically acceptable, Cryoprotectants must be able to penetrate into cells and tissues, and have low toxicity. Many compounds have such properties,
35 including glycerol, dimethyl sulfoxide, ethanediol, and propanediol, and the skilled

person will be aware of suitable cryopreservants and cryopreservation protocols (see, e.g. Pegg 2007).

In one embodiment hAECs are administered in a cryopreserved composition.

In one embodiment, the cryopreserved composition comprises sterile saline
5 containing human serum albumin (HSA) and dimethyl sulphoxide (DMSO).

In one embodiment, the cryopreserved composition comprises sterile saline containing 2% human serum albumin (HSA) and 0.16% dimethyl sulphoxide (DMSO) at a ratio of 3:1.

In another embodiment, the cryopreserved composition comprises sterile
10 saline containing 2% HSA and 0.32% DMSO at a ratio of 3:1.

Methods of Treatment

The methods described herein include treating a subject suffering from stroke or symptoms of stroke, by administering human amniotic epithelial cells (hAECs) at 2–
15 $8 \times 10^6/\text{kg}$ to the subject. Suitable administration methods are known in the art, and include intravenous infusion. In some embodiments, the hAECs are transfused intravenously. In some embodiments, administration of hAECs is by intravenous infusion divided over one or more days.

Cell therapy is a novel treatment modality, of interest especially to patients who
20 are unable to receive other forms of therapy, such as perfusion therapy, or for whom other treatments have failed (Phan et al. 2018). Without being bound by theory, cell therapy is typically based on the premise that the cells attenuate injury mechanisms and enhance the recovery process. The inventors of the present application have identified that stroke and stroke symptoms can be treated using hAECs.

25 In some embodiments, hAECs are administered to a subject by a number of cells relative to the weight of the subject to be treated, e.g., at about 2×10^6 cells, 3×10^6 cells, 4×10^6 cells, 5×10^6 cells, 6×10^6 cells, 7×10^6 cells or 8×10^6 cells per kilogram of the subject to be treated.

In one embodiment, the dose of hAECs is 2×10^6 cells/kg.

30 In one embodiment, the dose of hAECs is 4×10^6 cells/kg.

In one embodiment, the dose of hAECs is 8×10^6 cells/kg.

In one embodiment, the hAECs are given as one dose.

In one embodiment, the hAECs are infused in batches over several subsequent
days.

35 In one embodiment, the dose is maximally $2 \times 10^6/\text{kg}$ hAECs given intravenously each day over two days (a total of $4 \times 10^6/\text{kg}$).

In one embodiment, hAECs are administered by intravenous infusion over 1 hour.

In some embodiments, hAECs are administered over two or three divided doses, for example, where the dose to be administered is higher than a pre-determined threshold, for example higher than 4×10^6 cells/kg. In one embodiment, a dose of 4×10^6 cells is administered in two divided doses over two consecutive days. In another embodiment, a dose of 8×10^6 cells/kg is administered in three divided doses over three consecutive days. Divided dose may be of equal or unequal concentration of hAECs per dose.

In some embodiments the dose of hAECs to be administered is simply an absolute number of cells, e.g., said individual can be administered one or more doses of an amount in the range of about 100×10^6 cells to about 700×10^6 cells. In some embodiments, the dose to be administered as part of a single dose or total dose divided over 2 or more days is about 100×10^6 cells, 115×10^6 cells, 130×10^6 cells, 160×10^6 cells, 180×10^6 cells, 200×10^6 cells, 230×10^6 cells, 280×10^6 cells, 300×10^6 cells, 330×10^6 cells, 350×10^6 cells, 380×10^6 cells, 408×10^6 cells, 430×10^6 cells, 450×10^6 cells, 484×10^6 cells, 500×10^6 cells, 530×10^6 cells, 560×10^6 cells, 600×10^6 cells, 640×10^6 cells, 688×10^6 cells, or 700×10^6 cells.

In some embodiments, the hAECs are administered immediately or up to one, two, or three days post-stroke. In some embodiments the first of a sub-dose is given up to three days post-stroke.

In some embodiments, hAECs are given between 0 and 48 hours post-stroke.

In some embodiments, the total dose or the first sub-dose of hAECs is administered to a subject from between 0 to 24 and a second dose is administered to a subject from between 24 to 48 hours post-stroke.

In some embodiments, hAECs are administered up to seven days post-stroke. In some embodiments, the first of a sub-dose is given up to seven days post-stroke.

In some embodiments, the total dose or the first sub-dose of hAECs is administered to a subject immediately from post stroke up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27 or 28 days post-stroke.

In some embodiments, hAECs are administered after seven or more days post-stroke. In some embodiments, the first of a sub-dose of hAECs is given after seven or more days post-stroke.

In some embodiments, hAECs are administered any time from immediately post-stroke to any time as long as inflammation persists, for example, in one or several doses.

In one embodiment, the total dose or a first sub-dose of hAECs is administered to a subject whilst post-stroke inflammation is present. For example, whilst inflammation in the brain tissue of a subject is present.

5 *Combination therapies*

hAECs can also be used in combination with other agents of therapeutic value. In general, other agents do not necessarily have to be administered in the same pharmaceutical composition, and may, because of different physical and chemical characteristics, preferably be administered by different routes. The determination of the mode of administration and the advisability of administration, where possible, in the same pharmaceutical composition as the hAECs is well within the knowledge of the skilled clinician. The initial administration of other agents in combination with hAECs can be made according to established protocols known in the art, and then, based upon the observed effects, the dosage, modes of administration and times of administration can be modified by the skilled clinician.

hAECs and an additional therapeutic agent may be administered concurrently (e.g., simultaneously, essentially simultaneously or within the same treatment protocol) or sequentially, depending upon the nature and phase of the symptoms or condition to be treated, the condition of the patient, and the actual choice of therapeutic agents used. The determination of the order of administration of hAECs and any other additional treatment and the number of repetitions of administration of each therapeutic agent during a treatment protocol is well within the knowledge of the skilled physician after evaluation of the disease being treated, the condition of the patient, and the disclosures of the present application.

It is known to those of skill in the art that therapeutically-effective dosages can vary when the drugs are used in treatment combinations. Methods for experimentally determining therapeutically-effective dosages of drugs and other agents for use in combination treatment regimens are described in the literature. For example, the use of metronomic dosing, i.e., providing more frequent, lower doses in order to minimise toxic side effects, has been described extensively in the literature. Combination treatment further includes periodic treatments that start and stop at various times to assist with the clinical management of the patient.

For combination therapies, dosages of co-administered therapeutic agents will of course vary depending on the type of co-agents employed, the condition of the patient, and the condition to be treated.

Combination therapy may also include administration of hAECs in combination with surgical interventions. For example, mechanical or surgical thrombectomy is widely considered the gold standard for treating ischaemic stroke. Several animal models of stroke comprise aspects that simulate the mechanical thrombectomy, for example, withdrawal of the filament blockage in filament arterial occlusion.

In some embodiments, hAECs are administered prior to any other treatment.

In some embodiments, hAECs are administered simultaneously with or approximately contemporaneously with any other treatment,

In some embodiments, hAECs are administered after any other treatment.

In some embodiments, any other treatment may comprise several different treatments, which treatments may occur at different time points, and hAECs may be given prior to, contemporaneously or after any one or more of those treatments as either a single dose or as several doses and/or on several different occasions.

In some embodiments, the combination treatment comprises at least one of an anti-clotting agent, for example, aspirin, alteplase, Tenecteplase, or any other thrombolytic agent.

In some embodiments, the combination treatment comprises a mechanical intervention, such as a surgical intervention, for example, mechanical or surgical thrombectomy.

In one embodiment, hAECs are administered in combination with tissue plasminogen activator (tPA).

In one embodiment, tPA is administered first, and hAECs are administered subsequently to tPA administration.

In one embodiment, tPA is administered first, and hAECs are administered subsequently to tPA administration in one or several doses and/or sub-doses.

In one embodiment, tPA is administered from immediately after stroke and up to five hours post-stroke, and hAECs are administered after tPA administration.

In one embodiment, tPA is administered from immediately after stroke and up to five hours post-stroke, and hAECs are administered approximately 30 minutes after tPA administration.

The hAECs and additional therapeutic agent which make up a combination therapy disclosed herein may be a combined dosage form or in separate dosage forms intended for substantially simultaneous administration. The pharmaceutical agents that make up the combination therapy may also be administered sequentially, with either therapeutic compound being administered by a regimen calling for two-step administration. The two-step administration regimen may call for sequential

administration of the active agents or spaced-apart administration of the separate active agents. The time period between the multiple administration steps may range from, a few minutes to several hours, depending upon the properties of each pharmaceutical agent, such as potency, solubility, bioavailability, plasma half-life and kinetic profile of the pharmaceutical agent. Circadian variation of various physiological parameters may also be evaluated to determine the optimal dose interval.

In addition, administration or co-administration of a CIS inhibitor for treatment of an infection may be used in combination with procedures that may provide additional or synergistic benefit to the patient. By way of example only, patients may undergo genetic testing to identify genetic variation in their own genome or a pathogen's genome so as to optimize treatment parameters, e.g., the type of CIS inhibitor to be administered, dosing regimen, and co-administration with additional therapeutic agents.

Injectable Formulations

Formulations suitable for intramuscular, subcutaneous, or intravenous injection may include physiologically acceptable sterile aqueous or non-aqueous solutions, dispersions, suspensions or emulsions, and sterile powders for reconstitution into sterile injectable solutions or dispersions. Examples of suitable aqueous and non-aqueous carriers, diluents, solvents, or vehicles including water, ethanol, polyols (propyleneglycol, polyethylene-glycol, glycerol, cremophor and the like), suitable mixtures thereof, vegetable oils (such as olive oil) and injectable organic esters such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants. Formulations suitable for subcutaneous injection may also contain additives such as preserving, wetting, emulsifying, and dispensing agents. Prevention of the growth of microorganisms can be ensured by various antibacterial and antifungal agents, such as parabens, chlorobutanol, phenol, sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like.

hAECs migrate to injured tissue, including in the brain, and accordingly, need not be administered directly at the site of stroke injury or damage. It will be apparent to the skilled person that different administration routes have different technical requirements, for example, intravenous administration is limited by the amount of liquid that can be in patient circulation. The skilled person will also be aware that administration of intravenous cells is limited to quantities of cells that minimise the risk of cell aggregates or lumps of cells forming in the patient circulatory system. Other routes of

administration will necessarily allow modification of doses that are optimised for intravenous injection, which will be apparent to the skilled person.

In some embodiments, hAECs are given via intramuscular, subcutaneous, transmucosal or parenteral administration for stroke therapy in an amount in greater
5 than $8 \times 10^6/\text{kg}$. For example, intramuscular, subcutaneous, transmucosal or parenteral administration enables a dose of up to $9 \times 10^6/\text{kg}$, of up to $10 \times 10^6/\text{kg}$, of up to $11 \times 10^6/\text{kg}$, of up to $12 \times 10^6/\text{kg}$, and/or of up to $15 \times 10^6/\text{kg}$.

For intravenous injections, compounds may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution,
10 Ringer's solution, or physiological saline buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art. For other parenteral injections, appropriate formulations may include aqueous or nonaqueous solutions, preferably with physiologically compatible buffers or excipients. Such excipients are generally known
15 in the art.

Parenteral injections may involve bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The pharmaceutical composition may be in a form suitable for parenteral injection as a sterile suspensions, solutions, or
20 emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilising and/or dispersing agents. Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include
25 fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilisers or agents which increase the solubility of the compounds to allow for the preparation of
30 highly concentrated solutions. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

The pharmaceutical compositions may be in unit dosage forms suitable for single administration of precise dosages. In unit dosage form, the formulation is divided into unit doses containing appropriate quantities of one or more compound. The unit
35 dosage may be in the form of a package containing discrete quantities of the formulation. Non-limiting examples include aqueous suspension compositions packaged in single-

dose non-reclosable containers. Alternatively, multiple-dose reclosable containers can be used, in which case it is typical to include a preservative in the composition. By way of example only, formulations for parenteral injection may be presented in unit dosage form, which include, but are not limited to ampoules, or in multi-dose containers, with an
5 added preservative.

In some embodiments, provided is the use of up to about 8×10^6 /kg hAECs in the manufacture of a medicament for treatment of stroke or symptoms of stroke in a subject in need thereof.

In some embodiments, provided is a pharmaceutical composition comprising
10 about $2\text{--}8 \times 10^6$ /kg hAECs for the treatment of stroke and symptoms of stroke in a subject in need thereof, wherein the composition is administered up to three days post-stroke.

In some embodiments, provided is the use of hAECs in the manufacture of a medicament for treatment of stroke or symptoms of stroke in a subject in need thereof,
15 wherein the hAECs are to be administered at a dose in the range of about 100×10^6 cells to about 700×10^6 cells.

In some embodiments, provided is a pharmaceutical composition comprising about 100×10^6 to about 700×10^6 hAECs for the treatment of stroke and symptoms of stroke in a subject in need thereof, wherein the composition is administered up to three
20 days post-stroke.

EXAMPLES

Example 1: Mouse Models

Example 1.1: Materials and Methods

25 To investigate the efficacy of hAECs in reducing infarct volume and improving functional outcomes in different animal models of ischemic stroke, a trial protocol was established (Figures 1 and 2).

Accordingly, hAEC therapy is demonstrated to have improved outcomes even when administered up to three days after stroke onset, which is a significant advance
30 over previous treatments which required administration within the first 24 hours.

Statistical analysis

Data are presented as scatter plots combined with mean $\pm$ SEM, or scatter plot with median (neurological deficit scores). Analyses were performed using GraphPad
35 Prism (San Diego, CA; v6.0). Between-group comparisons were made using 1-way ANOVA or Student unpaired t test. If differences were detected by ANOVA, individual

groups were compared by Newman–Keuls or Tukey multiple comparison test. Neurological deficit scores were compared using a Mann–Whitney or Kruskal–Wallis test followed by Dunn test. Survival was analysed using a log-rank test. Two independent variables were compared using a 2-way ANOVA, followed by either
5 Bonferroni or Tukey test. Statistical significance was accepted if $P < 0.05$.

Image analysis

Images were de-identified prior to analysis and performed by JV. Segmentation of infarct was performed using MRlcroGL software (freely available at
10 <https://www.nitrc.org/plugins/mwiki/index.php/mricrogl:MainPage>).

Immunological assay

Laboratory studies of blood samples were performed to evaluate the effects of the hAECs on the immune response (highly sensitive C reactive protein, MMP-9, IL-6,
15 IL-10, IL-17, IFN-gamma, CD3, CD4, and CD19) post-stroke using pre-defined methods.

Example 1.2: Experimental models

Details of the animal models are shown in Figure 3. Most mice were subjected to either sham surgery or filament-induced right middle cerebral artery occlusion
20 (MCAO) for 30 minutes, and the filament was then retracted to allow reperfusion (transient MCAO). Filament-induced artery occlusion is a well-known and used model of ischaemic stroke. Some cohorts were subjected to 60 minutes of MCAO to confirm efficacy of hAECs after a stronger ischemic insult and reperfusion. Groups were only compared with others subjected to the same period of MCAO. Alternatively, the filament
25 was not retracted to induce permanent MCAO. Other mice were subjected to sham surgery or focal stroke of the left primary motor cortex induced by photothrombosis. In marmosets, stroke was produced in the left primary visual cortex by targeted intracortical injections of endothelin-1 proximal to the calcarine artery to cause transient arterial occlusion.

30

Example 1.3: Characterisation of hAECs

hAECs were isolated from term placentae donated by healthy volunteers who underwent elective Caesarean section delivery. In some mice, saline (vehicle) or 1×10^6 hAECs was injected into the tail vein either 1.5 or 72 hours after induction of MCAO. In
35 others, hAECs were injected at 1 and/or 14 days after photothrombotic stroke. In

marmosets, saline or hAECs (5×10^6) was injected into a saphenous vein 1.5 hours after initiation of ischemia.

Primary hAEC isolates were gated positive for epithelial cell marker epithelial cell adhesion molecule (EpCAM) and negative for mesenchymal stromal markers CD90 and CD105 (Figure 4). EpCAM+CD90-CD105- cells were further gated for stem cell and stromal markers. Primary hAECs were >95% negative for CD146, STRO-1, and CD44 and >90% positive for SSEA-4, CD29, and CD73 (Figure 4).

Example 1.4: Acute hAEC treatment limits functional deficit and infarct volume in transient MCAO.

Male mice that received hAECs (10^6 , IV) 1.5 hours after initiation of transient MCAO had less functional deficit at 24 and 72 hours than vehicle-treated mice. Specifically, hAEC-treated mice could grasp an elevated wire for approximately twice as long as vehicle-treated mice (Figure 15a), remove adhesive stickers from their forepaws quicker (Figure 15b), and had a lower median neurological deficit score (Figure 15c; $P=0.06$ at 72 hours). Infarct volume was reduced by $\geq 50\%$ in hAEC-treated mice, as evidenced by thionin staining and sequential within-animal magnetic resonance analysis at 24, 48, and 72 hours (Figures 9 and 15d).

Example 1.5: Migration of hAECs to brain and spleen is CXCR4 dependent in transient MCAO

hAECs labelled with carboxyfluorocin succinimidyl ester immediately before injection were detected in the ischemic hemisphere at 24 and 72 hours (Figure 16a and 16b) but not in the contralateral hemisphere (not shown) or in sham-operated mice (Figures 16a and b). hAECs were commonly localised to the infarct core (not shown) and spleen (Figure 16b). Few hAECs were in spleens of sham animals (Figure 16b), consistent with homing to multiple sites of injury and inflammation after stroke. Treatment of mice with CXCR4 antagonist, AMD3100, reduced hAECs by $\approx 70\%$ in the post-stroke brain (Figure 16c) accompanied by reduced grip strength and greater median neurological deficit score (Figure 16c), consistent with CXCR4/stromal cell-derived factor 1-mediated tissue homing. Interestingly, hAECs were ineffective at reducing infarct volume or functional deficits in splenectomised animals (Figure 16d), suggesting that the spleen is required for hAECs to elicit neuroprotection.

Example 1.6: hAECs limit stroke-induced cerebral apoptosis and inflammation in transient MCAO

Post-stroke brain expression of apoptotic markers was assessed using cleaved caspase-3, annexin V, and cleaved caspase-8. Immunofluorescence with the neuronal marker, NeuN, and cleaved caspase-3 confirmed that most apoptotic cells were neurons (Figure 17a). Administration of hAECs resulted in fewer apoptotic cells at 24 and 72 hours (Figures 5a and 5b and 17a), an effect most evident in the infarct core (Figures 5a and 15a). Expression of the extrinsic apoptotic pathway mediator, cleaved caspase-8, was also reduced in hAEC-treated animals (Figure 5c). In a primary neuronal culture-based model of stroke, hAECs exerted no direct protection of neurons subjected to glucose-deprivation injury (Figure 5d). Inflammatory processes contribute to cerebral infarct formation and resultant neuronal death. Because hAECs possess potent immunomodulatory properties, their effect on immune cell infiltration into the ischemic hemisphere was assessed using immunohistochemistry (Figure 17) and flow cytometry (Figure 6a). Increases in neutrophils, CD3+ T cells, and macrophages at 24 and 72 hours were blunted in mice treated with hAECs (Figures 6 and 17). Moreover, the proportion of 3-nitrotyrosine-positive macrophages (consistent with a proinflammatory phenotype) was markedly reduced at 72 hours in hAECs-treated mice (Figure 17d).

Flow cytometry confirmed these findings and revealed that ischemia-induced increases in B cells and CD8+ T cells were also blunted by hAECs (Figure 6a). Western blot analysis revealed that expression of the inflammasome protein, NOD-like receptor family pyrin domain-containing protein 3, was reduced in hAECs-treated animals at 24 hours (Figure 6b).

Example 1.7: hAECs modulate poststroke immunosuppression in transient MCAO

Flow cytometry was used to test whether treatment with hAECs affects stroke-induced systemic immunosuppression after early activation of the immune system. This phenomenon is an important cause of poststroke mortality and morbidity, the major hallmarks of which include splenocyte apoptosis, splenic atrophy, loss of splenic and circulating leukocytes, and a weakened type 1 T helper cell response. Indeed, the stroke-induced increase in apoptotic splenocytes evident by 24 hours preceded splenic atrophy at 72 hours, and these changes were blunted by hAECs (Figure 7a and b). Flow cytometry confirmed that stroke resulted in fewer leukocytes in atrophied spleens and circulating blood (monocytes, neutrophils, B cells, CD4+, and CD8+ T cells) and that these reductions were attenuated by hAECs (Figure 7c and Figure 8). Splenic expression of type 1 T helper cell (Stat4) and type 2 T helper cell (Stat6) transcription

factors was elevated at 72 hours, an effect prevented by hAECs (Figure VIIA in the online-only Data Supplement). A similar profile was seen for the Th17 transcription factor, Rorc (Figure 9a). Aerobic bacterial infection of lungs at 72 hours was unaffected by hAECs (Figure 9b).

5

Example 1.8: delayed treatment with hAECs promotes functional recovery in transient MCAO

To explore whether hAECs could impact functional recovery if administration was delayed for as long as 3 days—when infarct volume is fully formed in the mouse transient MCAO model (see Figure 15d), mice were administered hAECs at 3 days post-stroke. These mice exhibited greater grip strength (Figure 10a) and faster sticker removal at 7 to 14 days (Figure 10b) and tended to have a greater 14-day survival rate than mice receiving saline at 3 days (Figure 10c).

15 **Example 1.9: Acute administration of hAECs after permanent MCAO in young male mice improves outcome**

Acute administration of hAECs modestly blunted the impact of cerebral ischemia with no reperfusion on several functional and histological indicators, resulting in faster sticker removal, a tendency for greater grip strength and lower neurological deficit, and ≈20% smaller infarcts (Figure 18a and b). There were also fewer CD3+ T cells, but not neutrophils, within the ischemic hemisphere of hAECs- than vehicle-treated mice (Figure 18c and d).

25 **Example 1.10: Delayed treatment with hAECs promotes long-term functional recovery after photothrombotic stroke in aged female mice**

The efficacy of hAECs injected at 1 and/or 14 days poststroke to reduce motor deficit over 8 weeks in a cohort of aged (20–22 months) female mice subjected to photothrombosis- induced ischemia of the primary motor cortex was assessed. Motor function was assessed by grid-walking and cylinder tests every 7 days. Mice treated with hAECs at either 1 day only or 1+14 days exhibited reduced preference for using their unaffected (left) rather than affected (right) forelimb in the cylinder test at 8 weeks compared with vehicle-treated mice (Figure 19a). This equated to an ≈40% improvement in limb function for 8 weeks as a result of hAECs treatment at 1-day post-stroke, whereas no recovery occurred in vehicle-treated mice (Figure 19a). In the grid-walking test, ≈30% spontaneous recovery occurred in vehicle-treated mice by 8 weeks (Figure 19a).

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Similarly, mice treated with hAECs at 1±14 days made ≈40% fewer foot faults on their affected forepaw than vehicle-treated mice (Figure 19a). There was no apparent effect of hAECs on long-term motor function in either test if hAECs were administered only at 14 days (Figure 19a).

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Example 1.11: Delayed administration of hAECs stimulates repair mechanisms after photothrombotic stroke in aged female mice

A cohort of mice was studied to assess effects of hAECs administered 1-day poststroke on infarct development, peri-infarct plasticity, and/or the systemic inflammatory response at 7 or 56 days after photothrombotic stroke as potential mechanisms that impact long-term functional recovery. hAECs had no effect on infarct size at either 7 days (Figure 19b) or 56 days (Figure 11a). Changes in peri-infarct cortex plasticity were assessed by cell density, levels of microtubule-associated protein-2 (marker of neuronal dendrites), reactive astrogliosis (glial fibrillary acidic protein levels), and glutamic acid decarboxylase 67 (marker of inhibitory interneurons; Figures 11b, 12 and 19b). Administration of hAECs at 1 day resulted in a greater cell density and microtubule-associated protein-2 immunoreactivity in both regions 1 and 2 at 7 days compared with vehicle-treated controls (Figure 19b). Reactive gliosis, as measured by glial fibrillary acidic protein expression, was increased in region 1 and tended to be decreased in region 2 at 7 days in animals that received hAECs compared with vehicle (Figure 19b). At 56 days, hAECs-treated animals had more reactive astrocytes within the glial scar despite a similar glial scar area in vehicle controls (Figure 11b), and hAECs prevented the stroke-induced decrease in inhibitory interneurons (Figure 12).

When assessing effects on the systemic immune response, it was observed that hAECs reduced the expansion of myeloid cell compartments in bone marrow, spleen, and lymph nodes at 7 days (Figure 13a) and prevented the reduction of circulating B cells and expansion of B cells in spleen and lymph nodes (Figure 13b). hAECs also prevented the stroke-induced decrease in circulating T cells, without affecting T cell numbers in spleen or lymph nodes (Figure 13c).

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Example 1.12: hAECs treatment inhibits infarct expansion after endothelin-1-induced stroke in marmosets

Proof-of-concept studies were performed in 6 marmosets injected intravenously with either saline or hAECs (5×10⁶) 1.5 hours after initiation of cortical ischemia by injections of endothelin-1. Magnetic resonance analysis revealed infarct volume to be ≈70% and ≈125% larger on day 1 and day 10, respectively, in saline-

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versus hAECs-treated marmosets (Figure 19c). It was noteworthy that infarct volume expanded by >40% from day 1 to day 10 in saline-treated marmosets, whereas infarct expansion was largely arrested in hAECs treated animals (Figure 19c).

5 **Example 2: Combination therapies**

Example 2.1: Experimental design

Mice that underwent middle cerebral artery occlusion (MCAO) surgery were randomised into three treatment groups. Mice were injected intravenously (i.v.) with either: (1) vehicle only (200 μ L of 2% HSA in saline) after 30 min of reperfusion, (2) tPA (Alteplase, 10 mg/kg; Boehringer Ingelheim) at the start of reperfusion and then vehicle after 30 min, or (3) tPA at the start of reperfusion and then hAECs (1×10^6 cells in 200 μ L of 2% HSA in saline) after 30 min. A further 15 mice were sham-operated and then treated with vehicle (n = 7) or tPA + vehicle (n = 8) after 30 min. For sham surgery, we made a neck incision and exposed the CCA, ECA, ICA and bifurcation. No further surgical procedure or vessel ligation was made. Mice were euthanised according to designated endpoint times at 3 h (n = 21), 6 h (n = 31) or 24 h (n = 50) post-stroke. Animals euthanised at 24 h were assessed for functional deficits in two tests (see below). Brains were collected to determine infarct volume and assess markers of injury and inflammation.

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Middle cerebral artery occlusion (MCAO) model of stroke

MCAO surgery was performed as described (Evans et al., 2018). Briefly, mice were anaesthetised (ketamine 100 mg/kg and xylazine 10 mg/kg intraperitoneally; i.p.). Local anaesthetic (2 mg/kg bupivacaine; subcutaneously, s.c.) was injected in the incision area of the neck and rectal temperature was monitored and maintained at $37 \pm 0.5^\circ\text{C}$ throughout the surgery. The right common and external carotid arteries (CCA and ECA, respectively) were exposed. The external carotid artery was ligated and a vessel stump was made, thus permanently closing that artery after filament retraction. A small opening was made on the stump and a 6.0 silicone-coated monofilament was inserted through the opening and gently advanced into the internal carotid artery (ICA) to obstruct the origin of the right middle cerebral artery (MCA). The occlusion was maintained for 60 min, followed by filament withdrawal to allow reperfusion. Both successful occlusion (>65% reduction in cerebral blood flow; CBF) and reperfusion (>50% CBF to pre-ischemic level) were corroborated by transcranial Laser-Doppler flowmetry (Perimed). Neck and head incisions were then closed. All animals received 1 mL of sterile saline s.c. for hydration and returned to their cages after regaining consciousness.

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hAEC preparation for injection

Placentas were collected from healthy donors undergoing elective caesarean section for the delivery of a singleton birth. A total of 3 placentas were used in this study, with cells from only one donor administered to each mouse. Briefly, immediately after
5 delivery, the amniotic membrane was stripped from the chorion membrane of the placenta. The amniotic membrane was then repeatedly washed in Hanks' Balanced Salt Solution followed by digestion in trypsin solution. Isolated cells were filtered, pooled and assessed for purity (Murphy et al., 2010). Primary hAEC isolates were gated positive for the epithelial cell marker, epithelial cell adhesion molecule (EpCAM), and negative for
10 mesenchymal stromal markers CD90 and CD105 (Evans et al., 2018). Cells were cryopreserved in liquid nitrogen until use. Cells were thawed and washed with 2% human serum albumin (HSA) in saline to remove any excess dimethylsulfoxide (DMSO). The viability of cells post-thaw was assessed by trypan blue, and a cellular suspension of 5×10^6 cells/ml with viability $\geq 80\%$ was used.

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Functional assessment

At 24 h after MCAO, functional assessments were performed, as described (Evans et al., 2018). Clinical scoring was performed using a 6-point scoring system: 0 = normal motor function, 1 = flexion of torso and contralateral forelimb when mouse is
20 lifted by the tail, 2 = circling to the contralateral side when mouse held by the tail on a flat surface but normal posture at rest, 3 = leaning to the contralateral side at rest, 4 = no spontaneous motor activity, 5 = death prior scheduled endpoint. Latency to fall in the hanging grip test was used to assess grip strength. In this test, the time for which a mouse could remain suspended on a wire 60 cm above a soft surface was recorded,
25 and the average time of 3 trials with 2 min rest in between was calculated. The maximum time per trial was 180 s. All behavioural tests and further analyses were performed by an operator blinded to the group allocation of the animals.

Infarct analysis

30 At the endpoint of experiments (3 h, 6 h or 24 h post-stroke), mice were euthanised by inhalation of CO₂ and decapitation, and the brain was immediately removed, frozen in liquid nitrogen and stored at -80°C until sectioning. Coronal sections (30 μm) separated by $\sim 840 \mu\text{m}$ were obtained and stained with thionin (0.1%) to delineate the infarct (Jackman et al., 2009; Evans et al., 2018). Briefly, images of
35 sections were captured, and infarct area was measured manually using ImageJ software (NIH) in at least six 30 μm sections per brain that were spaced 840 μm apart by an

experienced researcher blinded to the treatment group. Infarct volume for each brain was then estimated based on these six measurements. Infarct volume was measured by applying the formula: corrected infarct volume = [left hemisphere area – (right hemisphere area – right hemisphere infarct area) x (thickness of section + distance
5 between sections)] (Jackman et al., 2009; Kim et al., 2014).

Immunohistochemical staining with chromogenic detection using 3,3'-diaminobenzidine (DAB) staining

DAB staining was performed to identify neutrophils. Briefly, 4%
10 paraformaldehyde (PFA) fixed coronal brain sections were blocked with peroxidase blocking solution (REALTM EnVisionTM Detection System, K5007, DAKO) for 10 min. After washing with phosphate-buffered saline (PBS), sections were blocked with 10% normal goat serum (NGS) for 40 min. Sections were rinsed and incubated with rabbit polyclonal anti-myeloperoxidase (MPO; 1:100; ab9535, Abcam) antibody overnight at
15 4°C. Following washing, sections were incubated with peroxidase-labelled polymer conjugated goat anti-rabbit immunoglobulin (DAKO) for 2 h at room temperature. Further, sections were washed and incubated with DAB reagent (DAKO) for 5 min in the dark, rinsed in distilled water and then counterstained with haematoxylin (1:4) for 30 s. Sections were then rinsed in Scott's tap water for 1 min and dehydrated through an
20 ascending ethanol series, immersed in xylene for 1 min and coverslipped with dibutylphthalate polystyrene xylene (DPX, Sigma) mounting medium. MPO+ neutrophils were counted by a researcher blinded to the sample identity.

Immunofluorescence

Coronal sections were stained to identify microglia/macrophages (ionised calcium-binding adapter molecule 1, Iba-1+), macrophages (F4/80+) and oxidative injury (3-nitrotyrosine; 3NT+). Human leukocyte antigen-G (HLA-G) antibody was used to detect the presence of hAECs in the brain, whilst IgG and matrix metalloproteinase-9 (MMP-9) were stained to assess for blood–brain barrier (BBB) dysfunction. Frozen brain
30 sections (10 µm) were fixed with ice-cold 4% PFA in 0.01 M PBS for 15 min. Then, sections were blocked with 10% NGS + 0.3% Triton X in PBS for 1 h, followed by overnight incubation with the following primary antibodies: rat monoclonal anti-F4/80 (1:50; ab6640, Abcam), mouse monoclonal anti-3-nitrotyrosine (anti-3NT; 1:50; ab61392, Abcam), rabbit polyclonal anti-ionised calcium-binding adapter molecule 1
35 (anti- Iba-1, 1:200; #17198, Cells Signalling Technology), mouse monoclonal anti-HLA-G (1:500; ab52455; Abcam) and rabbit anti- MMP9 (1:70, ab38898, Abcam). Double-

labelled immunostaining (F4/80 + 3NT) and HLA-G staining were performed as described (Evans et al., 2018; Kim et al., 2020). After washing, sections stained with a mouse antibody were processed according to the manufacturer's protocol using a Mouse-on-Mouse Kit™ (FMK-2201, Vector Laboratories) and the remaining sections
5 were incubated with a respective secondary antibody (goat anti-rabbit IgG Alexa 594 or goat anti-rat IgG Alexa 594, 1:500, ThermoFisher) for 2 h. Finally, sections were cover-slipped using Vectorshield mounting medium with 4,6-diamino-2-phenylindole (DAPI Vector Laboratories).

For IgG staining, 4% PFA-fixed slides were blocked with NGS and incubated
10 with Alexa 594 goat anti-mouse antibody (1:200, A-11032, Invitrogen) for 2 h. Then, sections were washed and coverslipped with Vectorshield mounting medium with DAPI. Brain sections were evaluated using the filters Cy3 (for F4/80, IgG, Iba-1 and MMP-9) or FITC (for 3NT and HLA-G). Cells positive for F4/80, 3NT and/or HLA-G were counted in the ischemic hemisphere and also the contralateral hemisphere in the case of MMP-
15 9. IgG and Iba-1 staining was assessed using ImageJ software. IgG was evaluated by intensity, and Iba-1 positive cells were counted per hemisphere section. Cells were quantified and images were captured using a light microscope (BX53, Olympus™) with a fluorescence lamp (X-Cite™ 120Q) attached, and with 20x or 40x objective lenses (UplanFL N, Olympus™). Images were taken in the ipsilateral hemisphere (infarct, peri
20 infarct and cortical regions) and corresponding locations in the contralateral hemisphere were included in the analyses. All sections were imaged on the same day using the same settings and analysed by a researcher blinded to the sample identity.

Statistical analysis

25 D'Agostino & Pearson or Kolmogorov–Smirnov normality tests were performed to assess distribution profiles of each variable in each data set. Normally distributed data were analysed using one-way ANOVA tests with Dunnett's post hoc test for multiple comparisons. Non-normally distributed or categorical data were analysed using Kruskal-Wallis tests. Mortality rate at 24 h was compared using the Chi-square test. Values are
30 expressed as mean $\pm$ standard error of the mean (SEM). All statistical analysis were performed using GraphPad Prism version 8.02 (GraphPad Software, CA, United States). Statistical significance was accepted when $p < 0.05$.

Example 2.2: Clinical score and functional deficits at 24 h after stroke

35 The initial cohort of mice designated for functional assessment and euthanasia at 24 h, the median clinical score was higher in those treated with tPA than in vehicle-

treated mice subjected to stroke (median score = 5 vs. 3, respectively; Figure 20a). This worsening of the 24 h post-stroke median clinical score by tPA alone was mainly due to a higher mortality (61%; 14/23) than in vehicle-treated controls (29%; 4/14, $p = 0.057$ vs. tPA + vehicle, Chi-square test; Figure 20a). Importantly, administration of hAECs in combination with tPA resulted in a lower mortality (27%; 4/15; $p = 0.039$ vs. tPA + vehicle, Chi-square test), indicating that hAECs exerted protective actions against severe adverse effects of tPA. The greater mortality in tPA-treated mice was related to ischemic stroke pathology because no mortality occurred over 24 h in an additional 8 sham-operated mice treated with tPA + vehicle (Figure 21).

As key endpoint measures (hanging grip time; fully established infarct volume) must be assessed in mice that survive for 24 h in this model of stroke (i.e., in those mice tending to have the mildest pathology), such different mortality rates between the treatment groups prevented a true and unbiased assessment of hAECs efficacy on these endpoint measures. Nevertheless, such data collected from these mice are shown in Figure 20b,c, indicating similar hanging grip performances and infarct volumes amongst mice that survived to 24 h.

Example 2.3: Infarct development

To investigate if early cerebroprotective effects of hAECs on infarct growth might be apparent before the period of mortality associated with tPA treatment (i.e., ≤ 6 h), infarct distribution and size were assessed at 3 h in additional groups of mice treated with vehicle, tPA + vehicle or tPA + hAECs, (all groups were $n = 7$) or 6 h ($n = 10, 11$ and 10 , respectively) after MCAO. No deaths occurred in any of these animals before the scheduled time for euthanasia (i.e., 3 h or 6 h). The data revealed substantial growth of the infarct core occurred between 3 h and 6 h after MCAO in mice treated with vehicle alone or tPA + vehicle, but not tPA + hAECs (Figures 22a–d). Furthermore, whereas there was no effect of tPA and/or hAECs on infarct volume at 3 h (Figure 22b), by 6 h it was apparent that infarct growth was augmented by ~50% by tPA + vehicle, but not by tPA + hAECs, in comparison to vehicle only (Figures 22c, d). Average increase in infarct volume from 3 h to 6 h was vehicle: 7.9 mm³ to 14.7 mm³ = 86%; tPA + vehicle: 8.1 mm³ to 23.2 mm³ = 186%; tPA + hAECs: 7.9 mm³ to 13.0 mm³ = 65%. Thus, infarct growth rate was 2-to 3-fold greater in mice treated with tPA + vehicle than in the other groups.

Example 2.4: hAEC localisation

Brain sections stained for HLA-G antibody revealed that hAECs injected i.v. at 1.5 h post-stroke were present in the ischemic hemisphere within 6 h (n = 9 mice, Figure 23). In each brain we examined six 10 µm sections, 420 µm apart, and found that the average number of hAECs in the ischemic hemisphere was 7.3 per section (Figure 23b). From these data and measurement of infarct length, we estimated that ~0.4% of hAECs administered (i.e., ~4,000 hAECs) had localised within in the ischemic hemisphere by 6 h post-stroke.

Example 2.5: Intracerebral haemorrhage

Intracerebral haemorrhage was visible in unstained brains from several mice, and perhaps especially in those treated with tPA + vehicle (see Figure 24a–c). To estimate the extent to which the BBB was injured, leading to bleeding into the brain parenchyma after stroke, brain sections were systematically stained for IgG. Immunofluorescence intensity revealed that BBB dysfunction in the ischemic hemisphere was augmented in mice treated with tPA + vehicle versus vehicle only (Figure 25a,b; p = 0.05); but was not augmented in tPA + hAEC-treated mice (Figure 25 a,b; p = 0.97 vs. vehicle only). This demonstrates suitability of hAEC treatment in haemorrhagic stroke, too, where treatment with tPA is unsuitable given it is a powerful blood thinner. Amongst all brains examined at 6 h post-stroke there was a significant positive correlation between infarct volume and IgG intensity, consistent with ischemic brain injury occurring in association with BBB disruption (Figure 24d). In brains from tPA + hAEC-treated mice, there tended to be a negative correlation between IgG intensity and HLA-G+ cells, consistent with the possibility that hAECs inhibited BBB breakdown (Figure 24e). MMP-9 was also assessed because its expression is related to BBB disruption, and we noted that even by 6 h following stroke there appeared to be a trend for more MMP-9-positive cells in the ipsilateral hemisphere of tPA + vehicle-treated mice than in mice treated with vehicle only (Figure 25c). However, there was no effect of hAEC treatment on the number of MMP-9-positive cells (Figure 25c).

Example 2.6: Inflammatory cells

To further explore potential mechanisms of hAEC-induced protection against infarct expansion in the presence of tPA, levels of inflammatory cells that may be associated with ischemic lesion formation in the mouse model of MCAO were assessed. No difference was found between groups in the number of microglia/macrophages (Iba+ cells; Figure 26a), neutrophils (MPO+ cells; Figure 26b) or macrophages (F4/80+ cells;

Figure 26c) in the ischemic hemisphere. However, there was a trend for fewer pro-inflammatory macrophages (F4/80+ 3NT+ cells) in the ischemic hemisphere of mice treated with tPA + hAECs compared with tPA + vehicle (Figure 26d, $p = 0.21$), consistent with a possible anti-inflammatory effect of hAECs. Representative images are shown in Figure 27.

Example 3: hAEC infusion at 7 days post-stroke provides long-term functional protection

A photothrombotic stroke model was used to induce an ischemic damage by means of photo-activation of a previously injected light-sensitive dye in 15-month old mice. Following illumination, the dye is activated to produce singlet oxygen that damages components of endothelial cell membranes, with subsequent platelet aggregation and thrombi formation, which eventually determines the interruption of local blood flow to simulate thrombotic stroke. Briefly, a photosensitive dye (Rose Bengal) was injected intraperitoneally to enter the blood stream. The dye was activated by a cold light source to induce endothelial damage with platelet activation and thrombosis, resulting in local blood flow interruption. The light source was applied on the intact skull with no need of craniotomy. The mice were sutured and allowed to wake up.

To explore whether hAECs could impact functional recovery if administration was delayed for as long as 7 days, mice were administered vehicle (control) or hAECs at 7 days after photothrombotic stroke and functional outcome was assessed over 56 days using the cylinder test for forepaw asymmetry ($n=9-10$). $**P<0.05$ (vehicle vs hAECs),

Statistical analysis was performed by 2-way ANOVA followed by Tukey' test. Administration of human amnion epithelial cells (hAECs) up to seven days post-stroke significantly improves long-term outcome after stroke in 15-month-old mice (Figure 28). Seven days in mice correlates to at least 28 days in humans, demonstrating efficacy of hAEC treatment in humans at least up to 28 days post-stroke. Without excluding other molecular mechanisms of action, this provides further support that hAEC treatment is effective for treatment of stroke for at least as long as inflammation persists.

Example 4: Human trials

Example 4.1 Trial protocol

Inclusion criteria

Patients were eligible if: (1) they had ischemic stroke in the territory of the large main artery (middle cerebral artery) (Phan et al., 2009); (2) presented within 24 h of

stroke onset and were not eligible for TPA or clot retrieval; (3) aged between 18 and 85 years old; and (4) had a National Institute of Health Stroke Scale/NIHSS score (tool used in clinical trials for measuring stroke severity) of between 6 and 15. Clinical characteristics and rationale for the treatment of the eight recruited patients (6 male) are provided in Table 1.

Table 1: Clinical characteristics and rationale for treatment of phase I clinical trial participants

Patient	Dose (x10 ⁶ cells/kg)	Weight (kg)	Age	Arterial Occlusion	Perfusion mismatch
1	2	90	60s	M2	0%
2	2	90	60s	M2	50%
3	2	86	70s	M2	50%
4	4	70	70s	A2	100%
5	4	102	70s	M2	50%
6	4	135 ⁺	40s	M2	50%
7	8	86	70s	M2	25%
8	8	119 ⁺	60s	M2	25%

10

Patient	TPA	NIHSS day 0	NIHSS day 7	NIHSS day 30	mRS 90 days	mRS 1 year
1	No	6	3	1	1	1
2	No	6	1	1	1	1
3	No	6	0	0	0	0
4	No (Apixaban)	7	12	16	4	4
5	No	6	mRS=3*	mRS=2*	0	n/a*
6	No	6	2	2	1	1
7	No (Dabigatran)	15	10	10	4	4
8	No	8	7	4	3	1

*Patient 5: Unable to perform NIHSS on day 7 and day 30 due to COVID-19 restrictions. mRS was instead performed via Telehealth; Unable to contact patient for 1 year follow-up.

+Patient dose was calculated by lean body weight. TPA = tissue plasminogen activator, M2 is distal segment of middle cerebral artery, A2 is distal segment of anterior cerebral artery

5 *Exclusion criteria*

Patients were excluded if there was evidence of: (1) autoimmune disease, organ transplant, malignancy, splenectomised individuals, or had infection at the time of stroke; (2) neurodegenerative disease such as dementia or Parkinson's disease; (3) pregnancy; (4) have contraindications for magnetic resonance (MR) imaging [patients with initial infarct (on DWI) volume <5 ml was excluded] (MR Stroke Collaborative Group et al., 2006); (5) mild stroke (NIHSS <6) or very severe stroke (NIHSS >15); and (6) eligible for TPA and/or ECR. During the course of the trial, we obtained an amendment from Monash Health Human Research Ethics Committee to include patients who had received TPA but less than 4 points reduction in NIHSS.

15

Treatment protocol

Doses of hAECs administered intravenously have ranged from 1×10^6 /kg in human infants with bronchopulmonary dysplasia (Lim et al., 2018) to $30\text{--}40 \times 10^6$ /kg in mice with experimental stroke (Evans et al., 2018a). Here, doses chosen for stage-wise escalation were: 2×10^6 , 4×10^6 , 8×10^6 , 16×10^6 , and 32×10^6 cells/kg. Patients were given hAECs by intravenous infusions over 1 h (Phan et al., 2018). At higher doses, patients were given hAECs over 2 or 3 divided doses (Table 2).

20

Sample size

This trial was designed to identify a safe dose range, including the maximum tolerated dose (O'Quigley et al., 1990). The dose escalation and de-escalation stages were designed in keeping with the principles of the classic 3 + 3 scheme in a phase I trial whereby the sample size is increased in small increments of 3. For a 5-dose escalation, the estimated sample size is 15 (Liu and Yuan, 2015).

30

Example 4.2: Safety of hAEC transfusion in stroke patients is safe (phase I dose-escalation trial)

Eight patients (six males) were recruited. Clinical characteristics and rationale for treatment are provided in Table 1. The mean age is 68.0 ± 10.9 . The frequencies of

hypertension and hyperlipidemia were 87.5%, diabetes was 37.5%, ischemic heart disease was 37.5%, atrial fibrillation was 50% and ever-smoker was 25%. The baseline NIHSS was 7.5 ± 3.1 , at 24 h was 7.8 ± 7.2 , and at 1 week was 4.9 ± 5.4 . The modified Rankin scale at 90 days was 2.1 ± 1.2 . Descriptive statistics were used to describe patient demographics, safety and SAE of hAECs. Regression analyses were not used given the eventual sample size of 8.

De-identified data will be available after two years. study protocol is available at ANZCTR trial website and in previous publication (Phan et al., 2018).

10 *Imaging characteristics*

Most of the patients in the study had more than 50% perfusion mismatch between cerebral blood volume and cerebral mean transit time. No patient was given alteplase due to the patient being outside of the time window or judged to have ischemic injury in at least 50% of the vascular territory. This decision was made by the treating clinicians. Another patient (patient 4) could not be given alteplase due to therapeutic apixaban level. Figure 29 illustrates the change in infarct volume over the first week. In seven of eight patients, there was no marked increase in infarct volume after cell infusion was completed. Patient 4 had recurrent stroke on the day after receiving hAECs and patient 7 had significant infarct growth prior to completion of hAEC infusions but without clinical deterioration in NIHSS. The NIHSS decreased from 15 on admission to 10 at 7 days (Table 1). Median infarct volume on FLAIR imaging at baseline was 11.74 ml (IQR 0, 39.89). Median ischemic volume on DWI at baseline was 29.10 ml (IQR 6.99, 42.92). Median infarct volume at post-treatment was 52.05 ml (UQR 22.2, 65.4). Median infarct volume at 1 week was 55.77 ml (IQR 37.26, 82.28).

25

Haematological profile

Haematological changes are displayed in Figure 30. There was no particular pattern observed among the 8 patients. Four patients had increases in white blood cells (WBC) above $10 \times 10^9/L$. The elevation in WBCs was transient in two of these four patients. The Chest XR, blood and urine cultures in these patients were normal. The haemoglobin profile did not dip below 120 g/L. The platelet count dipped below $100 \times 10^9/L$ in patient 7 on day 3 before returning to normal on day 7. The profile showed transient lymphopenia (below $1 \times 10^9/L$) in patient 4. Eosinophil counts were $0.10 \pm 0.10 \times 10^9/L$ (mean $\pm$ SD) at baseline and remained in the normal range at 5–7 days following cell infusion ($0.15 \pm 0.10 \times 10^9/L$, $n = 8$; data not shown).

35

Immunological profile

Immunological changes are displayed in Figures 31 and 32. There was no particular pattern observed among the 8 patients. In brief, the results show no obvious trends, especially in terms of effect of hAECs (e.g., between patients 7 and 8 that received the highest dose and patients 1–3 who received the lowest dose). We were not able to detect (or detected low levels) of several inflammatory markers (IFN-g, IL-6, TNF, IL-10, and IL-17). Levels of high sensitivity CRP and MMP-9 remained within normal range. In three patients (patients 1–3), MMP-9 levels did not change over the first week.

10 *Safety*

For the last four patients the infusion of hAECs was split across separate infusions on subsequent days to reduce risk for fluid overload (Table 2). This enabled an increase in dose of hAECs to 8×10^6 cells/kg. Supplemental oxygen was given in 5 patients during hAEC infusion (Table 3). These patients did not require oxygen therapy toward the end of the cell therapy procedure. Using pre-defined criteria, two serious adverse events occurred. One patient developed recurrent stroke 2 days after admission in the setting of atrial fibrillation (patient 4) and another patient developed pulmonary embolism 19 days later, whilst in rehabilitation (patient 7). No patient died within 1 year of trial enrolment. Overall, the data indicate that a dose of 2×10^6 /kg hAECs given intravenously each day over 2 days (a total of 4×10^6 /kg) is safe and optimal for use in a Phase II trial.

Table 2: Intravenous infusion of allogeneic hAEC in acute stroke patients

Patient	Dose (x10 ⁶ cells/kg)	Weight (kg)	Total dose (x10 ⁶ cells)	Infusion 1 Days post-stroke	Infusion 1 dose (x10 ⁶ cells)	Infusion 2 Days post- stroke	Infusion 2 dose (x10 ⁶ cells)	Infusion 3 Days post- stroke	Infusion 3 dose (x10 ⁶ cells)
1	2	90	180	1	180	NA	NA	NA	NA
2	2	90	180	1	180	NA	NA	NA	NA
3	2	86	160	1	160	NA	NA	NA	NA
4	4	70	280	1	280	NA	NA	NA	NA
5	4	102	408	1	204	2	204	NA	NA
6	4	135*	330	1	115	2	115	NA	NA
7	8	86	688	1	230	2	230	3	228
8	8	119*	484	1	161	1*	161	4*	162

NA = not applicable.

*Patient dose was calculated by lean body weight.

5 +Timing of the 2nd and 3rd infusions for Patient 8 were impacted by a shutdown of cell preparation facilities on Days 2 and 3 post-stroke.

Table 3: Adverse and serious adverse effects

Patient	Dose (x10 ⁶ cells/kg)	Age	AE1	AE1 Classification	AE1 Severity	AE2	AE2 Classification
1	2	60s	Supplemental Oxygen	Causal	Mild	NA	NA
2	2	60s	Supplemental Oxygen	Causal	Mild	NA	NA
3	2	70s	No	NA	NA	NA	NA
4*	4	70s	Fluid overload	Causal	Moderate	Infection	Probable
5	4	70s	Supplemental Oxygen	Causal	Mild	NA	NA
6	4	40s	No	NA	NA	NA	NA
7+	8	70s	Pneumonia	Probable	Moderate	NA	NA
8	8	60s	Supplemental Oxygen	Causal	Mild	NA	NA

Patient	AE2 Severity	AE3	AE3 Classification	AE3 Severity	SAE	SAE classification	SAE Severity
1	NA	NA	NA	NA	No	NA	NA
2	NA	NA	NA	NA	No	NA	NA
3	NA	NA	NA	NA	No	NA	NA
4*	Mild	Supplemental Oxygen	Mild	Mild	Recurrent Stroke	Probable	Severe
5	NA	NA	NA	NA	No	NA	NA
6	NA	NA	NA	NA	No	NA	NA
7+	NA	NA	NA	NA	Pulmonary Embolism	Probable	Severe
8	NA	NA	NA	NA	No	NA	NA

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as
5 illustrative and not restrictive.

All publications cited herein are hereby incorporated by reference in their entirety. Where reference is made to a URL or other such identifier or address, it is understood that such identifiers can change and particular information on the internet can come and go, but equivalent information can be found by searching the internet.
10 Reference thereto evidences the availability and public dissemination of such information.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of
15 these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed before the priority date of each claim of this application.

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CLAIMS

1. A method of treating stroke and/or symptoms of stroke in a subject in need thereof comprising administering human amniotic epithelial cells (hAECs) in an amount in the range of $2-8 \times 10^6/\text{kg}$ to the subject.
5
2. A method of treating stroke and/or symptoms of stroke in a subject in need thereof comprising administering human amniotic epithelial cells (hAECs) in an amount in the range of about $100-700 \times 10^6$ cells to the subject.
10
3. The method of claim 1 or claim 2, wherein the administration is by intravenous transfusion.
4. The method of any one of claims 1 to 3, wherein the dose of hAECs is about 2×10^6 cells/kg.
15
5. The method of any one of claims 1 to 3, wherein the dose of hAECs is about 4×10^6 cells/kg.
6. The method of any one of claims 1 to 3, wherein the dose of hAECs is about 8×10^6 cells/kg.
20
7. The method of any one of claims 1 to 3, wherein the dose of hAECs is an amount in the range of about 160×10^6 to about 230×10^6 cells.
25
8. The method of any one of claims 1 to 7, wherein the hAECs are given as one dose.
9. The method of claim 8, wherein the one dose is given over a period of about 1 hour.
30
10. The method of any one of claims 1 to 7, wherein the total dose of hAECs is divided into sub-doses and administered at two or more separate time intervals.
11. The method of claim 10, wherein the divided total dose is administered at dosage intervals over two or more subsequent days following the first dose.
35

12. The method of any one of claims 1 to 3, wherein the total dose is about 4×10^6 /kg hAECs administered intravenously in sub-doses over two consecutive days.
13. The method of any one of claims 1 to 12, wherein the hAECs are
5 administered in combination with another treatment or treatment regime.
14. The method of any one of claims 1 to 13, wherein the total dose or the first sub-dose of hAECs is administered to a subject from immediately post stroke up to three days post-stroke.
10
15. The method of any one of claims 1 to 13, wherein the total dose or the first sub-dose of hAECs is administered to a subject from between 0 to 48 hours post stroke.
16. The method of any one of claims 1 to 13, wherein the total dose or the first sub-dose of hAECs is administered to a subject from immediately post stroke up to 28 days post-stroke.
15
17. The method of any one of claims 1 to 16, wherein the hAECs are
20 administered in a cryopreserved composition.
18. The method of claim 17, wherein the cryopreserved composition comprises sterile saline containing 2% human serum albumin (HSA) and 0.16% dimethyl sulphoxide (DMSO) at a ratio of 3:1.
25
19. The method of claim 18, wherein the cryopreserved composition comprises sterile saline containing 2% HSA and 0.32% DMSO at a ratio of 3:1.
20. The method of any one of claims 1 to 19, wherein hAECs are positive for
30 EpCAM and negative for mesenchymal stromal markers CD90 and CD105.
21. The method of claim 20, wherein EpCAM+CD90-CD105- cells are further gated for stem cell and stromal markers.
22. The method of any one of claims 1 to 21, wherein hAECs express embryonic stem cell surface markers.
35

23. The method of claim 22, wherein the embryonic stem cell surface markers comprise one or more or all of:
- a) stage-specific embryonic antigen-3 (SSEA-3);
 - b) stage-specific embryonic antigen-4 (SSEA-4);
 - 5 c) octamer-binding transcription factor-4 (OCT-4);
 - d) tumour rejection antigen1-60 (TRA1-60); and
 - e) tumour rejection antigen 1-80 (TRA1-80)
24. Use of hAECs in the manufacture of a medicament for treatment of stroke or
10 symptoms of stroke in a subject in need thereof, wherein the hAECs are to be administered at a dose in the range of about 100×10^6 cells to about 700×10^6 cells.
25. Use of hAECs in the manufacture of a medicament for treatment of stroke or
15 symptoms of stroke in a subject in need thereof, wherein the hAECs are to be administered at a dose in the range of about 100×10^6 cells to about 700×10^6 cells.
26. The use of claim 20, wherein the medicament is administered up to seven days post-stroke.
- 20 27. A pharmaceutical composition comprising about $2-8 \times 10^6/\text{kg}$ hAECs for the treatment of stroke and symptoms of stroke in a subject in need thereof, wherein the composition is administered up to three days post-stroke.
- 25 28. A pharmaceutical composition comprising about 100×10^6 to about 700×10^6 hAECs for the treatment of stroke and symptoms of stroke in a subject in need thereof, wherein the composition is administered up to three days post-stroke.