An experimental platform for systemic drug delivery to the retina

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Degenerative retinopathies, including age-related macular degeneration, diabetic retinopathy, and hereditary retinal disorders—major causes of world blindness—are potentially treatable by using low-molecular weight neuroprotective, antiapoptotic, or antineovascular drugs. These agents are, however, not in current systemic use owing to, among other factors, their inability to passively diffuse across the microvasculature of the retina because of the presence of the inner blood–retina barrier (iBRB). Moreover, preclinical assessment of the efficacies of new formulations in the treatment of such conditions is similarly compromised. We describe here an experimental process for RNAi-mediated, size-selective, transient, and reversible modulation of the iBRB in mice to molecules up to 800 Da by suppression of transcripts encoding claudin-5, a protein component of the tight junctions of the inner retinal vasculature. MRI produced no evidence indicative of brain or retinal edema, and the process resulted in minimal disturbance of global transcriptional patterns analyzed in neuronal tissue. We show that visual function can be improved in IMPDH1−/− mice, a model of autosomal recessive retinitis pigmentosa, and that the rate of photoreceptor cell death can be reduced in a model of light-induced retinal degeneration by systemic drug delivery after reversible barrier opening. These findings provide a platform for high-throughput drug screening in models of retinal degeneration, and they ultimately could result in the development of a novel “humanized” approach to therapy for conditions with little or no current forms of treatment.

In transducing photons into electrical energy, the retina has the highest oxygen consumption per weight of any tissue in the human body (1). This, combined with a delicacy characteristic of all neuronal tissues, underlines a requirement for a unique and abundant blood supply capable of sustaining the retina’s energy demands while at the same time possessing efficient barriers, excluding potentially harmful bloodborne agents such as pathogens, antibodies, immune cells, and anaphylatoxins (2). There are two sources of blood supply to the retina: the central retinal artery, entering the retina at the optic disc, the arterioles of which terminate in both the inner plexiform layer and outer plexiform layer (OPL) and are readily visualized in images of the retinal surface, and the underlying choroidal circulation, providing nutrition primarily to the outermost layer of the retina, the photoreceptors (3). This outer layer of the retina remains avascular. The retina has two highly evolved and efficient barriers that maintain retinal homeostasis: the inner and outer blood–retina barriers (iBRB and oBRB, respectively). The iBRB comprises retinal endothelial cells, which line retinal microvessels, allowing for the maintenance of blood vessel integrity, preserving vessel homeostasis and mediating highly selective transport of molecules to the surrounding neural tissue (4). The oBRB is made up of a single layer of retinal pigment epithelial (RPE) cells situated at the back of the eye that act as a selective filter to restrict the passage of macromolecules to the outer segments of the retina (5). It is now widely accepted that up to 98% of low-molecular weight drugs cannot passively diffuse across these barriers (6), in part because of the highly evolved “tight junctions” (TJs) formed between adjacent endothelial cells or RPE cells. TJs are formed at the apical periphery of endothelial cells of the iBRB and RPE of the oBRB, and they perform the dual role of creating a primary barrier to the diffusion of solutes through the paracellular pathway while also maintaining cell polarity (7). TJs are complex and dynamic structures, composed of a series of integral and peripheral membrane proteins. The transmembrane proteins of the tight junction include occludin, junctional adhesion molecule, and claudins 1–24, proteins which extend into the paracellular pathway while also maintaining cell polarity (7). TJs are formed at the apical periphery of endothelial cells of the iBRB and RPE of the oBRB, and in the context of the current study it is of note that claudin-5 is absent from the TJs of the oBRB in adult mice (9). Claudins have been shown previously to interact homotypically with proteins on adjacent endothelial cells (10) and, in particular, claudin-5 knockout mice have altered blood–brain barrier (BBB) integrity (11). Although removal of claudin-5 compromised BBB function by allowing it to become permeable to molecules of up to ~800 Da, the barrier could still form, remaining intact and imperious to larger molecules, with fully formed TJs. These mice did, however, die within 10 h of birth, most likely because of the importance of claudin-5 in developmental processes. We have reported recently that levels of claudin-5 can be suppressed in adult mice by using an RNAi approach. Transient suppression of claudin-5 in the microvasculature of the brain causes a size-selective increase in passive diffusion of low-molecular weight molecules from the blood to the brain without any outward adverse detriment to such mice. Indeed, potentially pathological molecules far exceed the size-selectivity of barrier opening when claudin-5 is suppressed (12). Moreover, endogenous bloodborne molecules below 800 Da will be excluded from the brain and retina because of the presence of efflux transporters and specific receptors on the luminal surface of endothelial cells lining the microvasculature of the brain and retina (13).

Here, we report a preclinical and experimental platform technology allowing delivery of low-molecular weight therapeutics to the outer layers of the retina while excluding molecules greater than ~1 kDa. RNAi-mediated suppression of claudin-5 in the retinas of adult mice causes a transient and size-selective increase in paracellular permeability of microvessels without the development of retinal edema or changes in retinal function as assayed by electrophysiology. Moreover, we demonstrate the therapeutic potential of this technique by enhancing the delivery of two distinct low-molecular weight molecules to the neural retina of two rodent models of retinopathy: IMPDH1−/− mice (14), a model of auto-


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Suppression of Claudin-5 in the Retina.

Levels of expression of claudin-5 were significantly decreased 48 h after injection of siRNA targeting claudin-5 compared with the uninjected control, PBS control, and nontargeting (NT) control siRNA-injected mice. Suppression was transient, with levels returning to those observed in the control groups after 72 h (Fig. 1A and Figs. S1–S3). After retinal flatmount analysis of claudin-5 levels after delivery of siRNA, claudin-5 localization in retinal microvessels was manifested as a discontinuous pattern of staining 24 and 48 h after injection of claudin-5 siRNA compared with the control groups (Fig. 1B). These findings were in contrast to the continuous and intense pattern of staining of claudin-5 in the control groups and, indeed, at the 72 h time point after injection of siRNA, levels of claudin-5 in the retina appeared similar to the control groups, highlighting the transient nature of suppression. Although by Western blot analysis, levels of expression of claudin-5 appeared unchanged 24 h after siRNA injection, the pattern of staining in retinal flatmounts was not evident. Therefore, protein expression by Western blot at 24 h after claudin-5 siRNA injection more than likely represents immunoreactive yet non-TJ-associated claudin-5. Immunohistochemical analysis of retinal cryosections showed claudin-5 expression in vessels up and including the OPL, with levels decreased and mislocalized 24 and 48 h after injection of claudin-5 siRNA (Fig. 1C). Discontinuous and decreased expression of claudin-5 was not evident 72 h after injection. After suppression of claudin-5, electroretinographic (ERG) analysis of retinal function in mice 48 h after siRNA injection showed normal electrophysiological readouts in rod-isolated retinas from four mice (Upper) and decreased expression of claudin-5 was not evident 72 h after injection. Therefore, protein expression by Western blot at 24 h after claudin-5 siRNA injection more than likely represents immunoreactive yet non-TJ-associated claudin-5. Immunohistochemical analysis of retinal cryosections showed claudin-5 expression in vessels up and including the OPL, with levels decreased and mislocalized 24 and 48 h after injection of claudin-5 siRNA (Fig. 1C). Discontinuous and decreased expression of claudin-5 was not evident 72 h after injection. After suppression of claudin-5, electroretinographic (ERG) analysis of retinal function in mice 48 h after siRNA injection showed normal electrophysiological readouts in rod-isolated retinas from four mice (Upper). This finding was in comparison to standard mean ERG readings of between 800 μV after 48 h in uninjected mice (Lower).

**Results**

**Suppression of Claudin-5 in the Retina.** Levels of expression of claudin-5 were significantly decreased 48 h after injection of siRNA targeting claudin-5 compared with the uninjected control, PBS control, and nontargeting (NT) control siRNA-injected mice. Suppression was transient, with levels returning to those observed in the control groups after 72 h (Fig. 1A and Figs. S1–S3). After retinal flatmount analysis of claudin-5 levels after delivery of siRNA, claudin-5 localization in retinal microvessels was manifested as a discontinuous pattern of staining 24 and 48 h after injection of claudin-5 siRNA compared with the control groups (Fig. 1B). These findings were in contrast to the continuous and intense pattern of staining of claudin-5 in the control groups and, indeed, at the 72 h time point after injection of siRNA, levels of claudin-5 in the retina appeared similar to the control groups, highlighting the transient nature of suppression. Although by Western blot analysis, levels of expression of claudin-5 appeared unchanged 24 h after siRNA injection, the pattern of staining in retinal flatmounts was not evident. Therefore, protein expression by Western blot at 24 h after claudin-5 siRNA injection more than likely represents immunoreactive yet non-TJ-associated claudin-5. Immunohistochemical analysis of retinal cryosections showed claudin-5 expression in vessels up and including the OPL, with levels decreased and mislocalized 24 and 48 h after injection of claudin-5 siRNA (Fig. 1C). Discontinuous and decreased expression of claudin-5 was not evident 72 h after injection. After suppression of claudin-5, electroretinographic (ERG) analysis of retinal function in mice 48 h after siRNA injection showed normal electrophysiological readouts in rod-isolated retinas, with average readings of 841 μV in each eye from four mice (Upper). This finding was in comparison to standard mean ERG readings of between 800 μV after 48 h in uninjected mice (Lower).

**Figure 1.** Suppression of claudin-5 in retinal vasculature. (A) Western blot analysis of claudin-5 levels in the neural retinas of mice at 24, 48, and 72 h after injection of siRNA showed a significant decrease in levels 48 h after siRNA injection. ***, P = 0.0011. Western blots are representative of three independent experiments.** (B) Flatmount analysis of claudin-5 levels in the microvasculature of the neural retina showed decreased levels of expression and aberrant localization of claudin-5. At 24 and 48 h after injection of siRNA, the pattern of claudin-5 staining appeared diffuse and fragmented compared with the control groups. (40× objective.) (C) Claudin-5 expression was isolated to the retinal microvessels, processing as far as the OPL of the retina. At 24 and 48 h after injection of claudin-5 siRNA, levels of claudin-5 appeared mislocalized and fragmented. (40× objective.) (D) ERG analysis of adult C57Bl6 mice revealed no distinct changes in ERG tracings 48 h after injection of claudin-5 siRNA, with mean ERG readings of 841 μV in each eye from four mice (Upper). This finding was in comparison to standard mean ERG readings of between 800 μV after 48 h in uninjected mice (Lower).
was not observed in the control groups of mice, nor was it observed at the 72-h time point after injection of claudin-5 siRNA. Gd-DTPA has a molecular mass of 742 Da and will not freely passively diffuse across microvessels into the retina in homeostatic circumstances. In a sagittal perspective, levels of Gd-DTPA were observed to infiltrate the retina in large quantities at the 48-h time point after siRNA injection compared with NT siRNA control mouse, with densitometric analysis of mice showing significantly increased levels of contrasting agent in the eyes of mice 48 h after injection of claudin-5 siRNA ($P = 0.0018$; Fig. 2C, double asterisk).

The size-selective nature of transient modulation of the iBRB was readily observable when a molecule of $\sim 1,900$ Da was perfused in mice after claudin-5 siRNA injection. Microperoxidase (molecular mass, 1,881 Da) was detected in the microvessels of the retinas of all mice after all experimental treatments. In conditions where the iBRB or BBB is damaged, microperoxidase will extravasate in vast quantities, yet this was not the case when claudin-5 was suppressed (Fig. 2D). Size-selective barrier opening observed, as well as being a transient process, did not appear to cause any neuronal cell death in any layer of the retina of mice after analysis of DNA fragmentation in retinal layers after siRNA delivery (Fig. 2E).

**Enhanced Delivery of GTP to the Retinas of IMPDH1$^{-/-}$ Mice.** Given our observations of maximum suppression of claudin-5 levels 48 h after injection of siRNA, this was chosen as the optimum time point at which to assess efficacy of therapeutic systemic delivery of compounds to the retina. IMPDH1$^{-/-}$ mice lack the rate-limiting enzyme for the de novo synthesis of GTP, a central mediator of visual phototransduction. With increasing age, these mice develop a mild retinopathy, as evidenced by decreases in electrophysiological readout from the retina in tandem with a thinning of the photoreceptor outer nuclear layer (ONL) in animals older than 10 months (14). GTP has a molecular mass of 523 Da and will not freely passively diffuse across the iBRB if injected systemically. Indeed, we have previously attempted to inject GTP intraocularly to induce increases in ERG in IMPDH1$^{-/-}$ mice ($n = 4$), but without success (Fig. 3A). At 48 h after injection of siRNA, levels of claudin-5 were significantly decreased in IMPDH1$^{-/-}$ mice (Fig. 3B). Suppression was manifested by a distinct change in the pattern of staining observed in retinal cryosections compared with mice receiving NT siRNA (Fig. 3C). Before experimentation, retinal function of all IMPDH1$^{-/-}$ mice was assessed by ERG, and 48 h
before injection of GTP, mice were injected with either an NT or claudin-5-targeting siRNA. Subsequently, after injection of GTP i.p., retinal function was assessed immediately by ERG, and a highly significant increase in rod-isolated b-wave amplitude was observed in those mice that had received claudin-5 siRNA (n = 6) compared with the NT control (n = 8) group (Fig. 3D). Analysis of cone-isolated ERG after enhanced delivery of GTP did not show any significant differences between the experimental groups (Fig. 3E). ERG tracings revealed well-formed a and b waves in mice after enhanced systemic delivery of GTP to the retina (Fig. 3G), in contrast to mice receiving no iBRB modulation (Fig. 3F), where no change in ERG output was observed.

Enhanced Delivery of ALLM to the Retinas of Light-Exposed BalB/c Mice. Light-induced retinopathy in albino BalB/c mice has been used previously to induce photoreceptor cell apoptosis. Indeed, many antiapoptotic agents have been screened in vivo by using this technique but by direct intracocular injection of these agents before light ablation (17). Ca\(^{2+}\) activated calpains have been shown to be associated with neuronal cell death in different neuropathological diseases, including retinopathy (18). Calpains are often associated with necrotic cell death; however, they are also found to be activated during apoptosis (19). We chose to assay the effects of enhanced systemic delivery of a known calpain inhibitor, \(\gamma\)-acetyl-Leu-Leu-Met-CHO (ALLM). This molecule has a molecular mass of 401 Da and is a potent inhibitor of both calpain I (\(K_i = 120\) nM), calpain II (\(K_i = 230\) nM), cathepsin-L (\(K_i = 0.6\) nM), cathepsin-B (\(K_i = 100\) nM), and the proteasome (20). It does not readily passively diffuse across the iBRB and has been used previously to demonstrate efficacy in preventing photoreceptor apoptosis when intraocularly injected (17).

Forty-eight hours before injection of ALLM (20 mg/kg), mice received an injection of either an NT siRNA or a claudin-5 siRNA. Mice were dark-adapted for a further 24 h after injection of ALLM, after which time they were exposed to 6,000-lux white light for 2 h. Mice were returned to the dark room, and 12, 24, and 48 h after light ablation, photoreceptor nuclei were analyzed for TUNEL for identification of end-stage dying cells. With enhanced systemic delivery of ALLM, at 12 h (\(P = 0.0011\); Fig. 4A, ***) and 24 h (\(P = 0.0252\); Fig. 4B, *) after light exposure, the retinas of BalB/c mice showed significantly fewer TUNEL-positive cells in the ONL of the retina, directly implying less photoreceptor cell death. ALLM slows the rate of cell death, because 48 h after light exposure (Fig. 4C), the number of TUNEL-positive cells increased in the group of mice receiving ALLM subsequent to claudin-5 siRNA (*, \(P = 0.0191\)). Mice analyzed at 4 days and 7 days after light ablation showed no differences in TUNEL-positive cells in the ONL (Fig. 4D).

**Discussion**

Although degenerative retinopathies, including age-related macular degeneration (AMD), diabetic retinopathy, and RP represent major causes of world blindness, treatment options are currently exceedingly limited (15, 21–24). No form of prevention is available for the nonexudative (dry) form of AMD, apart from dietary intervention. With regard to the exudative (wet) form of AMD, intraocular injection of the monoclonal antibody Lucentis (Genentech Inc.), targeting VEGF, is being used in a growing number of cases; however, severe endophthalmitis, resulting in blindness, has been reported to occur in up to 2% of treated patients (25). Proliferative diabetic retinopathy is treatable by scatter and focal laser surgery, but the procedure itself induces significant retinal damage and can involve in excess of 1,000 laser burns to the retinas of diseased...
and direct intraocular injection (Fig. 3). Euthanasia was performed in animals older than 10 months. GTP has a molecular mass of 523 Da, with a half-life of about 10 minutes. Retina in tandem with a thinning of the photoreceptor ONL in the retina and also for the neural retina resulted in a significant increase in the rod-isolated ERG function in these mice because of a low rate of diffusion of the compound across the iBRB. Indeed, photoreceptor cell death did not manifest in the claudin-5 siRNA-injected mice until 48 h after light exposure. These findings display enhanced delivery of ALLM to the retina before being rapidly hydrolyzed.

As a method of enhanced delivery of low-molecular weight therapeutics to the retina, RNAi-mediated suppression of claudin-5 may offer a therapeutic potential in this condition. Claudin-5 plays a role in the formation of paracellular pores or channels that function in mediating selective ion permeability (11, 32). However, the function of claudin-5 in the retina appears to be more dynamic than simply structural. It has recently been reported that mice exposed to hypoxic conditions exhibit down-regulation of claudin-5, which selectivity disrupts the iBRB, allowing passage of small molecules into the retina, similar to the phenotype seen in claudin-5-deficient mice (33). Moreover, levels of claudin-5 in the brain appear to fluctuate depending on environmental stimuli, such as exposure to alcohol, traumatic brain injury, and stroke (34–36).

To explore the therapeutic potential of transient compromise in barrier integrity, we sought to deliver therapeutic molecules to the retinas of two mouse models of retinopathy. First, we chose a model of autosomal recessive RP, the IMPDH1−/− mouse (14). These animals lack one of the two rate-limiting enzymes of de-novo synthesis of GTP, a central mediator of visual phototransduction (15). With increasing age, these mice develop a mild retinopathy, as evidenced by decreases in electrophysiological readout from the retina in tandem with a thinning of the photoreceptor ONL in animals older than 10 months. GTP has a molecular mass of 523 Da, and direct intraocular injection (Fig. 3) did not have an impact on ERG function in these mice because of a low rate of diffusion of the compound across the iBRB and limited diffusion across intact cell layers of the inner retina. However, when levels of claudin-5 were suppressed, enhanced systemic delivery of GTP to the outer layers of the retina resulted in a significant increase in the rod-isolated ERG response from IMPDH1−/− mice in comparison with those injected with NT siRNA and ALLM. **P < 0.0011. At 24 h after light ablation, the number of TUNEL-positive cells in mice that received claudin-5 siRNA and ALLM was significantly decreased compared with mice receiving NT siRNA and ALLM.

Second, we explored the efficacy of enhanced delivery of an antiapoptotic agent to the retinas of mice before light-induced ablation of photoreceptors. Albino BalB/c mice exposed to varying degrees of intense white light are very susceptible to photoreceptor cell death, due in part to activation of calpains in photoreceptors of the retina (37). To study the effects of increased delivery of ALLM on photoreceptor cell survival, we injected 20 mg/kg of the inhibitor intraperitoneal 24 h before light exposure with and without iBRB modulation. TUNEL staining became apparent 12 h after the initial exposure to light; however, significantly less cell death was observed in the group of mice that received ALLM when the iBRB was modulated with claudin-5 siRNA compared with NT siRNA. ALLM has a molecular mass of 401 Da and will have a low rate of diffusion across the plasma membranes of photoreceptors, and enhanced delivery will simply allow for increased quantities of GTP to access the neural retina before being rapidly hydrolyzed.

![Fig. 4. Enhanced delivery of a calpain inhibitor to retinas of light-exposed BalB/c mice.](image-url)

Enhanced delivery of a calpain inhibitor to retinas of light-exposed BalB/c mice. Effects of either an NT or claudin-5 siRNA together with a potent calpain inhibitor, ALLM, on light-induced retinal degeneration were assessed in BalB/c mice. Claudin-5 or NT siRNA was administered to BALB/c mice 48 h before injection of 20 mg/kg ALLM. Mice were dark-adapted for 24 h before being exposed to white fluorescent light of 7,900 lux for 2 h. (A) At 12 h after light ablation, TUNEL-positive nuclei present in the ONL of retinal sections of mice injected with claudin-5 siRNA and ALLM were significantly fewer than those injected with NT siRNA. **P < 0.0011. (B) At 24 h after light ablation, the number of TUNEL-positive cells in mice that received claudin-5 siRNA and ALLM was significantly decreased compared with mice receiving NT siRNA and ALLM. *, P < 0.052. (C) There were significantly more TUNEL-positive nuclei in retinas of claudin-5 siRNA- and ALLM-injected mice compared with NT siRNA- and ALLM-injected mice at 48 h after light exposure. **P < 0.0191. (40x objective.)
can now be used in animal models of retinopathy to screen a wide range of potentially therapeutic molecules ranging from antiapoptotic agents to antiangiogenic compounds. Not only does suppression of claudin-5 have little impact on neuronal homeostasis, we have also shown that ETV6 transcriptional dysregulation causes global transcriptional perturbations in neuronal tissue when the BBB is transiently modulated (Tables S1 and S2). This indicates that rather than “breaking” the BBB and iBRB, targeted suppression of claudin-5 selectively modulates barrier function while not causing neuroinflammatory activation, edema, or cell death. Indeed, coupled with commercially available reagents, such as in vivo-jetPEI (Polyplus-transfection), regular barrier opening in experimental rodents could be used for longer-term studies pertaining to drug delivery. This, however, would not be the case in “humanizing” this delivery system, and for the purposes of systemic delivery of drugs targeting degenerative retinal conditions in humans, localized barrier opening could, in principle, be limited to retinal tissues by incorporating barrier-opening machinery into an inducible viral delivery system. In this regard, it is of note that adeno-associated virus (AAV) delivery systems have now been approved for use in a growing number of gene therapy trials, including those recently initiated for the congenital retinopathy Leber’s congenital amaurosis (38–40), and recent studies have indicated that AAV9 is capable of targeting gene therapy trials, including those recently initiated for the congenital retinopathy Leber’s congenital amaurosis (38–40), and recent studies have indicated that AAV9 is capable of targeting vascular endothelial cells, rendering the concept of an inducible AAV9 system for barrier opening potentially feasible (41).

Materials and Methods

Animal Experiments and Experimental Groups. All studies carried out in the Ocular Genetics Unit at Trinity College Dublin (TCD) adhere to the Association for Research in Vision and Ophthalmology statement for the use of Animals in Ophthalmic and Vision Research. Mice were sourced from Jackson Laboratories and bred on site at the Ocular Genetics Unit in TCD. Extensive Materials and Methods are outlined in SI Text.


In Vivo Delivery of siRNA to Retinal Capillary Endothelial Cells. Rapid high-pressure, high-volume tail vein injections were carried out essentially as successfully used previously at this laboratory (12, 42, 43). Antibodies for Western blots were as follows: polyclonal rabbit anti-claudin-5 (1:500, Zymed Laboratories) and polyclonal rabbit anti-j-actin (1:1,000; Abcam). Membranes were washed with Tris buffered saline (0.05 M Tris, 150 mM NaCl, pH 7.5) and incubated with a secondary anti-rabbit (IgG) antibody with HRP conjugates (1:2,500, Sigma–Aldrich), or with anti-mouse (IgG, 1:1,000; Sigma–Aldrich), for 3 h at room temperature. Immune complexes were detected by using ECL. All Western blot analyses were repeated a minimum of three times (n = 3 mice per treatment).

ERG Analysis of C57/B16 and IMPDH1−/− Mice. Extensive details are outlined in SI Text.

Assessment of iBRB Integrity by Perfusion of Tracer Molecules. After RNAi-mediated ablation of transcripts encoding claudin-5, microperoxidase (5 μg body weight of 25 mg/ml microperoxidase (MP-11, 1,862 Da Sigma–Aldrich) was perfused at 37 °C through the left ventricle of the beating heart of anesthetized mice 24, 48, and 72 h after hydrodynamic delivery of claudin-5 siRNA.

MRL. After injection of siRNA and by using appropriate controls, iBRB integrity to a molecule of 742 Da (Gd-DTPA) was assessed via MRI using a dedicated small rodent Bruker BioSpec 70/30 magnet system (i.e., 7-T field strength, 30-cm bore diameter) with an actively shielded USR magnet.

TUNEL Analysis. Data after TUNEL staining were expressed as mean TUNEL-positive cells per section ± SEM, as determined by an individual blinded to the treatment. Results were analyzed by using a two-tailed Student’s t-test, with P < 0.05 considered significant.

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