Alcoholism: Clinical and Experimental Research

Vol. 44, N
October

ALCOHOLISM CLINICAL & EXPERIMENTAL RESEARCH

The Synergistic and Neuroprotective Effects of Alcohol–Antioxidant Treatment on Blood–Brain Barrier Endothelial Cells

David Fisher, Kelly Angelique Thomas, and Sahar Abdul-Rasool

Background: Alcohol (EtOH) is reported to adversely affect one of the most crucial roles of the blood–brain barrier (BBB), the regulation of its permeability, thereby compromising the stability of the homeostatic environment of the brain. The central component of the BBB, endothelial cells (ECs), regulates BBB transcellular transport, while their paracellular pathways are made virtually impermeable by molecular structures called tight junctions (TJs). These TJs are composed of proteins, such as claudin-5, a protein involved in the regulation of paracellular permeability and of key interest in this study.

Methods and Results: The working hypothesis of this study postulated that the high levels of antioxidants (AOs) in the fermented *Aspalathus linearis* (Rooibos; Rf) tincture may protect the ECs of the BBB against oxidative stress induced by EtOH exposure. Cells were exposed for 24 hours to selected concentrations of EtOH (25 and 100 mM), Rf (containing an antioxidant equivalence of 1.9 nM Aspalathin), and cotreatments of EtOH and Rf. Cell viability, live cell number, and toxicity were analyzed using the trypan blue exclusion assay. RT-qPCR was implemented to quantify claudin-5 transcription. In addition, permeability (Transepithelial Electrical Resistance) of bEnd5 monolayers was measured. The experimental timeline for the above-mentioned parameters was 24 and 48 hours.

Conclusions: Our study showed that simultaneous exposure of Rf and EtOH was able to negate the effects of EtOH on cell viability and cell proliferation, but was not able to reverse or reduce the effects of EtOH on claudin-5 transcription and paracellular permeability. Furthermore, a novel finding in this study suggests that very low concentrations of AOs in tinctures such as Rooibos tea could profoundly alter the redox status of brain ECs.

Key Words: Blood, Brain Barrier, Alcohol, Brain Endothelial Cells, Claudin-5, Aspalathin, Antioxidants.

CRITICAL TO MAINTAINING brain homeostasis is the blood–brain barrier (BBB). It serves as a vascular interface protecting the central nervous system (CNS), by preventing the entry of toxic blood-borne substances into the brain microenvironment (Colgan et al., 2008), and regulates the movement of molecules and ions between the blood and the brain's parenchyma. The transport of these substances across the capillary endothelial cells (ECs) of the brain is mostly regulated via transcellular pathways, and in general, the paracellular pathways are sealed using tight junctions (TJs), which are located apically on the lateral walls of adjacent ECs (Fisher and Mentor, 2020). Small molecules that are nonpolar and lipophilic are able to transverse across the BBB with ease along their concentration gradients, and influence the physiological function of the endothelial component of the BBB. Molecules such as nicotine, methamphetamine, and alcohol have these attributes and can easily pass through the BBB and impact neural systems (Gabathuler, 2010; Pardridge, 1995). However, surprisingly the metabolism of these narcotics within the ECs has been reported to produce increased reactive oxidant species (ROS) production, which impacts the crucial regulatory function of the BBB (Abdul Muneer et al., 2011; Haorah et al., 2005a, 2005b).

ROS are small, extremely reactive, oxygen-containing molecules, naturally produced in minute quantities during metabolic reactions in the body and, under normal physiological conditions, are neutralized by specialized endogenous antioxidants (AOs), such as glutathione, catalase, and superoxide dismutases (SOD). When produced in excess, they pose consequential health risks. Alcohol stimulates the activity of alcohol dehydrogenase and cytochrome P450-2E1 (Haorah et al., 2008a, 2008b), which accelerates ROS production, as well as diminishes the levels of endogenous AOs (Wu and Cederbaum, 2003). Alcohol-induced ROS affects the TJs, which are responsible for sealing the paracellular pathways across the endothelial cell layer (Abdul Muneer et al., 2011). Studies have shown that a chief mechanism influencing BBB permeability is the ROS-induced redistribution and/or the

From the Department of Medical Biosciences, (DF, KAT, SA-R), University of the Western Cape, Cape Town, South Africa; and School of Health Professions, (DF), University of Missouri, Columbia, Missouri.

Received for publication April 5, 2020; accepted July 23, 2020.

Reprint requests: David Fisher, Department of Medical Biosciences, University of the Western Cape, Private Bag X17, Bellville 7535, Cape Town, South Africa; Tel.: +27-21-959 2185; Fax: +27-21-959 1339; E-mail: dfisher@uwc.ac.za, davidwfisher333@gmail.com

DOI: 10.1111/acer.14433

suppression of the tight junction protein claudin-5 (Schreibelt et al., 2007). The organization of other tight junction proteins, occludin and ZO-1, are also suppressed by exogenous ROS in brain ECs (BECs; Schreibelt et al., 2007).

Endogenous AOs are cellular substances capable of neutralizing excess ROS, which may otherwise contribute to the formation of oxidative stress (OS; Berker et al., 2013; Dai and Mumper, 2010). They are radical scavengers that disrupt and inhibit radical chain reactions (Huang et al., 2009). AOs have been proposed to delay or inhibit diseases such as Alzheimer's disease, cancer, and coronary heart failure (Berker et al., 2013). Exogenous AO supplements, such as vitamins C and E, as well as foods and teas with high AO concentrations, are commonly used as a therapy for common illnesses such as colds and flus (Poljsak et al., 2013).

Of key interest in our study is the popular beverage (*Aspalathin linearis*) known both locally and internationally as Rooibos (red bush), which contains high concentrations of a well-known antioxidant, Aspalathin (Joubert and Schulz, 2006; Krafczyk and Glomb, 2008; Mentor and Fisher, 2017; Pengilly et al., 2008; Snijman et al., 2009; Standley et al., 2001).

Here, we report on the neuroprotective ability of the potent *Aspalathin linearis* tincture (Rf), in concentrations which are similar to plasma concentrations found after a typically consumed dosage (in Rooibos tea) by the general population, on the alcohol-induced oxidative damage on the in vitro BBB model. We also examine the effects of low concentrations of Rf on cultured BECs, and furthermore, we report for the first time on the potential of Rooibos-derived AOs to nullify or reverse the effects of EtOH on the ECs of BBB.

MATERIALS AND METHODS

The study was aimed at evaluating whether the detrimental effects of acute alcohol treatment (24-hour exposure) on the brain endothelial cell line, bEnd5, could be avoided or reversed by cotreatment with concentrations of a fermented Rooibos (Rf) aqueous extract containing 1.9 nM of Aspalathin (Mentor and Fisher, 2017). Our concentration of Rf was selected based on the reported peak plasma concentration of Aspalathin after the consumption of 500 ml of the Rf tincture (Breiter et al., 2011).

Bioreagents

Cell culture reagents included Dulbecco's modified Eagle medium (DMEM-Ham's F-12, Whitehead Scientific, Cat No. 12-719F), supplemented with 1% antibiotic penicillin/streptomycin–amphotericin B mixture (Whitehead Scientific (Pty) Ltd, Cape Town, South Africa, Cat No. 13-115E), 1% sodium pyruvate (GIBCO®, Cat No. 11360), 1% nonessential amino acids (NEAAs; Lasec, Cat No. 13-114E), and 10% fetal bovine serum (FBS; Separations, SV30160.02). Phosphate-buffered saline (PBS; Cat No. D8662), trypan blue (0.4%; Cat No. T-8154), and hydrocortisone (Cat No. H0888) were purchased from Sigma-Aldrich, Modderfontein, South Africa, while trypsin (0.25%; Cat No. BE 02-007E) and dimethyl sulfoxide (DMSO; Cat No. sc-358801) were purchased from Whitehead Scientific (Pty) Ltd.

Culture of bEnd5 Cells

Mouse BECs (bEnd5; ECACC, Cat No. 96091930) were grown in sterile tissue culture-treated flasks until 70 to 80% confluence in DMEM (Whitehead Scientific, 12-719F) supplemented with 10% FBS (Separations, SV30160.02), 1% NEAAs (Lasec, 13-114E), 1% sodium pyruvate (Whitehead Scientific, 13-115E), and 1% antibiotic (pen–strep; Whitehead Scientific, 17-745E). For assays, cells were trypsinized at confluence using 0.25% EDTA–trypsin (Whitehead Scientific, BE02-007E).

Trypan Blue Exclusion Assay

For the trypan blue assay, mouse BECs (bEnd5; ECACC, Cat No. 96091930) were seeded at a density of 50,000 cells in clear 6-well tissue culture plates ($n = 6$) and were incubated for 24 hours to allow for attachment. Thereafter, the ECs were exposed to 25 and 100 mM EtOH (Sigma, Modderfontein, South Africa, E7023), Rf containing equivalence of 1.9 nM Aspalathin (South African Rooibos Council, Batch No. P06/02KK), and combinations of 25 mM EtOH and Rf, and 100 mM EtOH and Rf, respectively. After 24 hours of exposure to EtOH/Rf, all media were replaced with untreated DMEM. The cells were then incubated for the selected time intervals between 24 and 96 hours. Cells were trypsinized at 80 to 90% confluence using 0.25% EDTA–trypsin.

An established ratio was used (10 μl of cell suspension, 60 μl complete DMEM, and 30 μl trypan blue dye). 10 μl of this cell suspension was then added to the hemocytometer and observed under a standard inverted phase-contrast microscope for cell counts. The % cell viability was determined by dividing the number of live cells by the total number of cells multiplied by 100. Similarly, % cell toxicity was determined by dividing the number of dead cells by the total number of cells multiplied by 100.

Transcription Analysis: RNA Extraction of bEnd5 Cells

For RNA extraction and subsequent PCR analysis, bEnd5 ECs were seeded at a density of 150,000 cells in clear 90-mm tissue culture-treated petri dishes ($n = 6$). Cells were treated as per the above protocols with 25/100 mM EtOH, Rf (containing 1.9 nM Aspalathin), and cotreatments with EtOH and Rf. Once cultures reached 80 to 90% confluency, they were rinsed twice with 1 ml PBS (Lonza, Whitehead Scientific, BE17-516F). 1 ml of cold Tripure (Roche, 11667165001) was then added to each petri dish plated with exposed cells at the respective time interval and homogenized. Each millilitre of homogenate was transferred to a 2-ml Eppendorf tube and incubated for 5 minutes at room temperature. 0.2 ml of chloroform (Kimix, SA, chl001) was added to the homogenate and vortexed for 15 seconds. This was then incubated for 5 to 10 minutes at room temperature. The solution was separated into 3 phases by centrifuging at 12,000 *g* for 15 minutes. 400 μl of the upper aqueous phase was transferred to a 1.5-ml Eppendorf tube. Subsequently, 0.5 ml of isopropanol (Kimix, SA, ipr001) was added to the solution and mixed thoroughly. RNA precipitated overnight at −20°C. RNA was then centrifuged at 12,000 *g* for 10 minutes and the supernatant removed. Following this, 1 ml of 75 to 80% molecular EtOH was added to the RNA pellet, vortexed, and centrifuged at 7,500 *g* for 5 minutes. The supernatant was aspirated, and the pellet was resuspended in 100 μl DEPC-treated water (Invitrogen, AM 9916). The quality of the RNA samples was interrogated using the NanoDrop ND-1000 (Thermo Scientific, ND1000). The control RNA samples were electrophoresed on a 1% agarose gel to determine the quality.

cDNA Synthesis

A starting concentration of 2 μg RNA was used for cDNA synthesis. For each sample, 2 reactions were carried out. A reverse

transcriptase buffer, random primers, dNTP mix, MultiScribe™ Reverse Transcriptase (High-Capacity cDNA Synthesis Kit; Life Technologies, Waltham, MA, USA, 4368814), and nuclease-free water (BIO-37080 Bioline Water, 18.2 MΩ PCR Grade) were added to 10 μl of RNA sample up to a total reaction volume of 20 μl. The components were thoroughly mixed and spun down using a benchtop centrifuge to collect all the liquid. Cycling was performed on the GeneAmp® PCR System 9700 (Life Technologies). The synthesized cDNA was stored at −20°C until further processing. Before expression analysis, each experimental sample was diluted 1:5 with nuclease-free water (BIO-37080 Bioline Water, 18.2 MΩ PCR Grade).

Several control samples were cleaned using NaOAc/ethanol (EtOH) precipitation. Briefly, 2.5 volume of ice-cold 100% EtOH was added to each sample, with 1/10 volume of 3 M NaOAc. The samples were incubated at −80°C for 2 hours. Thereafter, the samples were centrifuged for 30 minutes at 4°C to precipitate the RNA. The supernatant was removed and the pelleted RNA washed 1× with 70% ice-cold EtOH. The samples were air-dried for 10 minutes and resuspended in TE buffer.

Gene Expression Analysis

For gene expression analysis, each reaction (for standard curves and samples) consisted of 1 μl cDNA template (equivalent to approx. 200 ng RNA), 0.125 μl of each of the primers (final concentration of 125 nM), 5 μl Power SYBR® Green PCR Master Mix (Life Technologies, 4367659), and nuclease-free water. Expression analysis was performed on the ABI 7900HT Fast Real-Time PCR System followed by a dissociation (melt) curve analysis. Postcycling, the data were analyzed using the SDS v2.3 software (Life Technologies) and relative expression analysis performed using qBase + (BioGazelle).

Two genes (occludin and claudin-5) were tested. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine-guanine phosphoribosyltransferase (HPRT) were used as housekeeping genes to check the integrity of the samples. The primer sets used were synthesized by Whitehead Scientific, and their sequences are included in Table 1. Twelve RNA samples (all controls) were electrophoresed on a 1% agarose gel to determine the quality. Two ribosomal RNA species were observed for most samples.

Table 1. Primer Information

Gene (*Mus Musculus*)	Primer sequence	Accn no	Trnscrpt length (bp)
Occludin	Forward primer: 5′-GTT GAA GAG CAA ATT ATC-3′ Reverse primer: 5′-TTA GAG TCC AAA GTC AG-3′	NM_008756	3,193
Claudin-5	Forward primer: 5′-GGG TGA GCA TTC AGT CTT TA-3′ Reverse primer: 5′-CAG CAC AGA TTC ATA CAC CT-3′	NM_013805	1,420
HPRT	Forward primer: 5′-AGT CCC AGC GTC GTG ATT AG-3′ Reverse primer: 5′-TCC AAA TCC TCG GCA TAA TG-3′	NM_013556.2	1,349
GAPDH	Forward primer: 5′-GTC GGT GTG AAC GGA TTT G-3′ Reverse primer: 5′-TGG CAA CAA TCT CCA CTT TG-3′	NM_001289726	1,296

Accn no (accession number); Trnscrpt length (transcript (mRNA) length).

An aliquot of cDNA was synthesized from 12 control bEnd5 samples. The 12 control samples were treated using NaOAc/EtOH precipitation (Ambion AM9740, Kimix, SA, UN1170) and were pooled and then used to generate an 8-point, 2-fold serial dilution series in order to determine the efficiency of the primer sets. In addition, cDNA from *MusMusculus* spleen RNA samples were used as an external control to generate an 8-point, 2-fold serial dilution series. GAPDH and HPRT amplified in the bEnd5 control samples and spleen samples indicating that the RNA was intact and functional. Regarding the experimental genes, MusMusculus occludin were then tested on the aforementioned samples. The standard curves were generated by plotting the Ct value of each serial dilution point against the known quantity of cDNA in each serial dilution. Each standard curve was generated in triplicate for each data point. All amplification curves were submitted for a melt-curve analysis to evaluate primer specificity. No primer–dimers or nonspecificity was observed in HPRT, GAPDH, or claudin-5. Table 2 shows PCR amplification efficiency for the target genes. Finally, the relative value was measured.

Seventy-two (72) RNA samples were submitted for gene expression analysis of 2 target genes (claudin-5 and occludin) relative to 2 reference genes (HPRT and GAPDH). The quality of the qPCR raw data was interrogated using SDS v2.3 (Life Technologies) and outliers removed. qBase+ (BioGazelle) was utilized to determine the expression of the target gene relative to the reference genes. Each experimental sample was assayed in triplicate to account for technical variation. Occludin was not expressed at any time point, in any treatment group, and thus excluded from analysis.

Transepithelial Electrical Resistance (TEER)

Across confluent monolayers of BECs (bEnd5), TEER was measured using an Ohm Millicell-ERS (Electrical Resistance System; Millipore, MERS 000 01), which measures the transmembrane resistance of the epithelia in culture. The TEER measurements of the blank inserts (no cells) were subtracted from the readings that were obtained from the inserts with confluent endothelial monolayers so that the true resistance (resistance of the cell monolayer) could be determined. The resistance of the cell monolayer was multiplied by the surface area of the insert used to grow the monolayer of cells (0.6 cm^2) to calculate the resistance normalized per square cm ($\Omega.cm^2$). The resistance between the blank inserts containing only media and the blank inserts containing media with the respective experimental compounds was identical. Therefore, blank inserts consisted of media with the respective experimental compounds. The controls were implemented by growing confluent endothelial monolayers. These control monolayers were not exposed to any of the experimental compounds. The culture media were supplemented with 10% FBS, as TEER values using this concentration of FBS remained consistent for longer in addition to aiding the proliferation of cells (Derk et al., 2015).

1×10^6 bEnd5 cells (Sigma, 96091930) were seeded per well on sterile inserts (Microsep, PIHA012) and grown to confluence for 24 hours in clear 24-well tissue culture plates ($n = 6$). Samples were

Table 2. cDNA Synthesis Cycling Parameters

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time	10 minutes	120 minutes	5 minutes	∞

then exposed to 25 and 100 mM EtOH (Sigma, E7023), Rf containing equivalence of 1.9 nM Aspalathin, and combinations of 25 and 100 mM EtOH together with Rf, respectively, for 24 hours. Following the 24-hour exposure to the compounds, the various compounds were removed from the cells and replaced with untreated DMEM (Whitehead Scientific, 12-719F) and were further incubated for 48, 72, and 96 hours.

The electrodes were immersed in the wells, and once stable resistance readings were observed, the TEER values were recorded. Readings were taken in duplicate every 2 hours at 3 time intervals.

Statistical Analysis

MedCalc, Ostend, Belgium (version 11.6.1) was used to statistically analyze the data obtained. Normality was determined using the Kolmogorov–Smirnov test. The Student *t*-test (for normally distributed independent samples) and Mann–Whitney (for not normally distributed independent samples) test were applied, and the probability of $p < 0.05$ was designated as statistically significant.

RESULTS

Effects of EtOH on Live Cell Number

The adverse and acute effects of alcohol on the BECs are well established in the literature (Haorah et al., 2005a, 2005b; Mandyam et al., 2017; Singh et al., 2007). However, the extended effects over a period of 48 hours on BECs following a single 24-hour exposure have not previously been described in the literature.

EtOH (25 and 100 mM) decreased the number of live Bend5 cells in culture at both 24 and 48 hours ($p < 0.01$). The percentage suppression of 25 mM EtOH at 24 hours (26.5%) was exacerbated at 48 hours to 33%, whereas the percentage suppression by 100 mM EtOH was higher at 24 hours (37.5%), and it decreases to 33% at 48 hours (Fig. 1*A*).

Effects of Rf on Live Cell Number

Treatment with the extremely low dose of Rf had no discernible effects over the first 48 hours ($p > 0.05$; see Fig. 1*B*). Compared to control cell numbers, the means of Rf-treated Bend5 cells were not significantly different at both 24 and 48 hours (Fig. 1*B*).

Concurrent Effects of EtOH and Rf on Live Cell Number

EtOH have been reported in the literature to bring about its effects on BECs by effects by inducing elevated ROS. We examined whether concurrent treatment of alcohol with Rf, which has high levels of AOs (Mentor and Fisher, 2017), could ameliorate the ROS-inducing effects of EtOH.

Cotreatment of Bend5 cells with EtOH (25 and 100 mM) and Rf completely reversed the suppressive effects of EtOH at both 24 and 48 hours (Fig. 1*C*). There were no statistical differences between control numbers of live cells and those treated concurrently with EtOH and Rf ($p > 0.05$).

Effects of EtOH and Rf on Cell Viability

2It was important to determine whether the suppression of cell proliferation was due to the toxicity of the EtOH. The parameter, cell viability %, is an indicator describing the number of live cells relative to dead cells within a population of cultured cells, with high percentages indicating high numbers of live cells relative to dead cells and vice versa.

Control viability (%) of Bend5 cells was statistically not different from Bend5 cells treated with 25 and 100 mM EtOH ($p > 0.05$) at both 24 and 48 hours (Fig. 1*D*). Control viability (%) was not different from Bend5 cells treated with Rf at 24 and 48 hours ($p > 0.05$; Fig. 1*E*).

Compared to controls, treating Bend5 ECs concurrently with 25 mM EtOH and Rf had no statistical effect at 24 hours ($p > 0.05$). However, for Bend5 cells cotreated with 100 mM EtOH and Rf, a small, but statistically significant ($p < 0.05$) decrease in viability (5.36%) relative to control viability was observed. At 48 hours, cotreatment at both 25 and 100 mM EtOH produced no statistical difference to control viability ($p > 0.05$; see Fig. 1*F*).

Based on the low numbers of dead cells for both EtOH- or RF-treated cells, as ascertained by the trypan blue–based viability assay (see Fig. 1*D*/*E*), it was unlikely that the suppression of cell numbers was caused by the toxicity of the EtOH.

Effects on Transendothelial Permeability (TEER)

Effects of EtOH on Transendothelial Permeability. Although the literature has reported that alcohol increases the permeability of the BBB and that it down-regulates the TJ expression, this is the first report on the effects of acute (24-hour) treatment on transendothelial permeability extending over 96 hours. Control TEER followed a typical profile for Bend5 endothelial monolayer, which saw a steady increase in TEER between 24 and 72 hours, plateauing at 96 hours. In contrast, both concentrations of EtOH, 25 and 100 mM, significantly ($p < 0.05$) decreased TEER at 24 hours by an average of 20.4% and at 48 hours by an average of 57.24% ($p < 0.01$). However, TEER did not recover to control levels at either 72 hours or 96 hours (average of 42.2 and 48.2% below control levels, respectively; $p < 0.01$), indicating an increase in the permeability across the BEC monolayer throughout the 96-hour time frame of the experiment (see Fig. 2*A*). Both concentrations of EtOH had similar effects on the permeability across the monolayer, and there was no discernible pattern, which resembles a dose–response effect. These experiments indicated that a short exposure (24 hours) to alcohol may induce long-term effects on the permeability of the BBB.

Effects of Rf on Transendothelial Permeability. Acute exposure to Rf (see Fig. 2*B*) also had similar effects in significantly decreasing TEER by an average of 48.6% ($p < 0.01$), thereby implying an increase in the permeability across

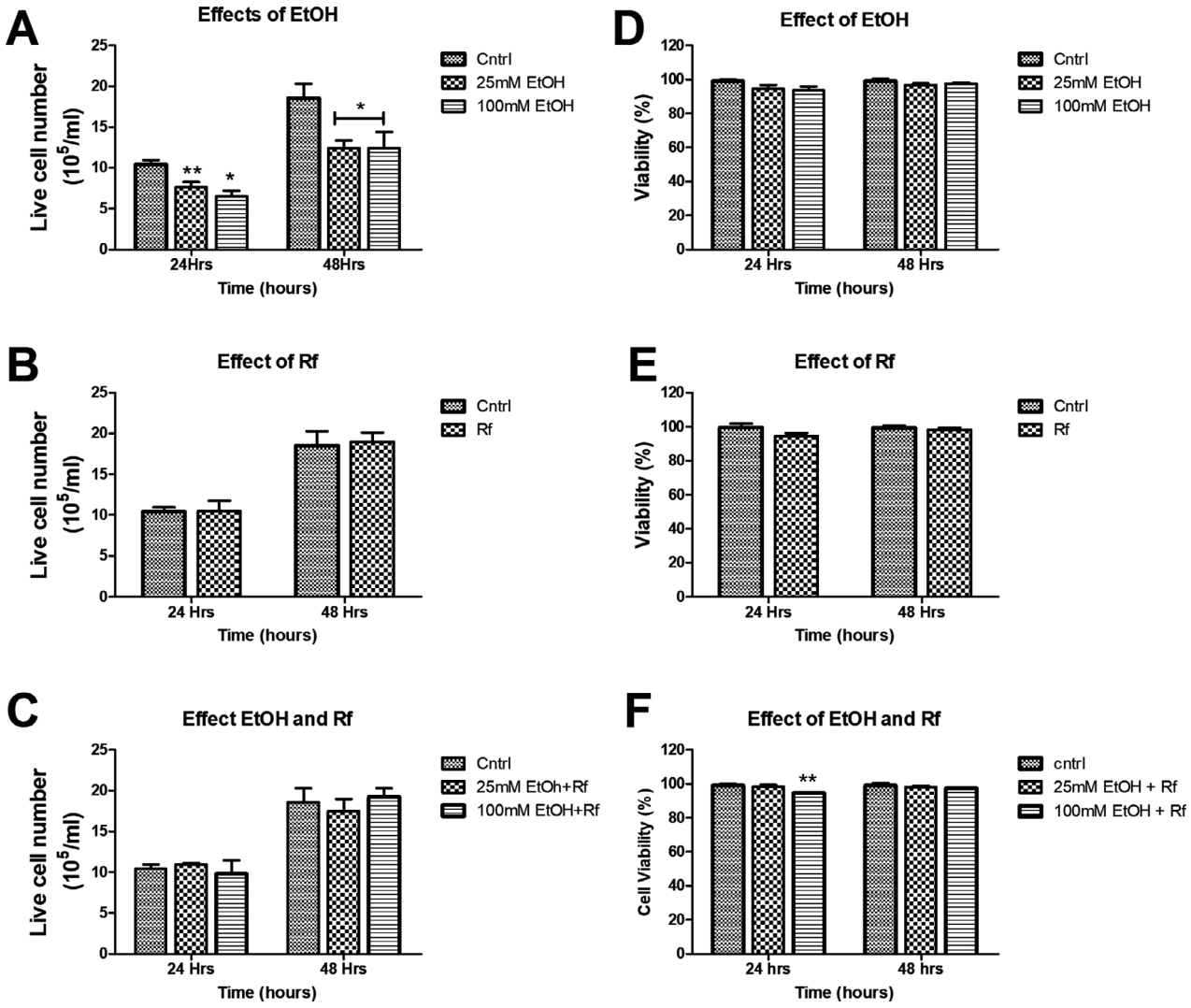

Fig. 1. The effects of EtOH and Rf on live cell number and viability. (**A**) The graph represents the suppressive effects of EtOH exposure on bEnd5 brain endothelial cell division over 48 hours. (**B**) Rf had no statistical effects on bEnd5 brain endothelial cell live cell number. (**C**) Combinations of EtOH and Rf (after 24-hour exposure) reversed the effects of EtOH on bEnd5 brain endothelial cell numbers. (**D**) There were no statistical effects of 25 and 100 mM on bEnd5 EC viability as ascertained using the trypan blue assay. (**E**) There were no statistical effects of Rf on bEnd5 EC viability. (**F**) A small decrease in viability was brought about by the combination of 100 mM EtOH and Rf. *$p < 0.01$ and **$p < 0.05$ designate statistical significance compared to controls ascertained using Student's *t*-test. Data are displayed as mean ± SEM ($n = 6$).

confluent layers of BECs. The suppression of TEER by Rf was not statistically different between 24 and 96 hours ($p > 0.05$).

Effects of Cotreatment of Alcohol and Rf on Transendothelial Permeability. Cotreatment of bEnd5 cells with both alcohol and Rf had no effect on reversing the EtOH-induced decreases in TEER (Fig. 2*C*). At 24 hours, compared to controls, the combined treatment decreased TEER (25 and 100 mM) by an average of 34.6%, whereas at 48 hours TEER for both 25 and 100 mM was reduced further to an average of 72.9%. TEER for both 25 and 100 mM recovered slightly at 72 and 96 hours to an average of 48.9% below levels of the control (Fig. 2*C*).

Effects on TJs

The expression of TJs plays a crucial role in the regulation of permeability across the paracellular pathways between adjacent ECs in brain capillaries. Claudin-5, one of the most influential TJ proteins, which is thought to be primarily responsible for the impermeability of the brain capillary paracellular pathways, was used as a molecular marker in determining TJ integrity. We tested whether 24-hour exposure to EtOH, Rf, and cotreatment of these would have any effect on the expression of claudin-5 over a period of 48 hours.

Effects of EtOH on Claudin-5 Transcription. The data reflect a very dynamic transcriptional response to the 24-

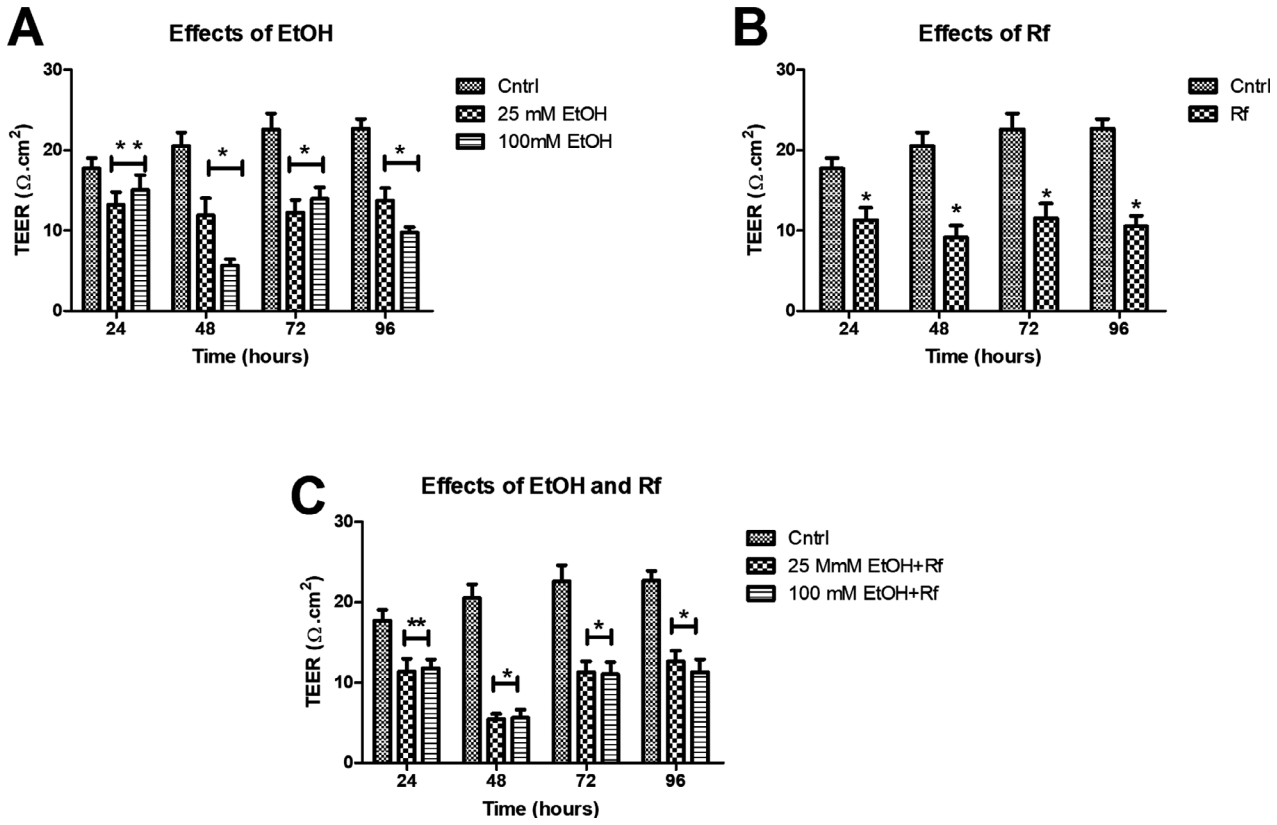

Fig. 2. Effects of EtOH and Rf on permeability. (**A**) The effects of 25 and 100 mM EtOH on TEER following a 24-hour exposure ($n = 6$). (**B**) The effects of Rf on TEER following a 24-hour exposure ($n = 6$). (**C**) The effects of combinations of EtOH and Rf, respectively, on TEER following a 24-hour exposure. *$p < 0.05$ denotes statistical significance compared to controls ascertained using the Mann–Whitney test. Results were displayed as mean ± SEM ($n = 6$).

hour treatment with both concentrations of EtOH. However, claudin-5 expression responded to the EtOH treatment differently: At 24 hours, the lower EtOH concentration (25 mM) produced nonstatistical ($p > 0.05$) suppression of the claudin-5 transcription, while the 100 mM treatment produced a 4-fold elevation in transcription ($p < 0.01$). At 48 hours, in the 25 mM EtOH-treated Bend5 cell population, the levels of claudin-5 increased to control transcription levels, while the higher concentration of EtOH (100 mM) was severely suppressed ($p < 0.05$; see Fig. 3*A*).

The Effect of Rf on Claudin-5 Transcription—Exposure to Rf increased the transendothelial permeability of the BECs (Fig. 2*B*). Our PCR experiments endorsed this finding by showing significant decreases in claudin-5 expression across the 48-hour time frame of the experiment (Fig. 3*B*). Thus, the decrease in claudin-5 TJ protein seems to be responsible for the increase in transendothelial permeability. This also indicates that the primary mechanism for Rf effects may be by increasing the paracellular pathway permeability.

Effects of Cotreatment of EtOH and Rf on Claudin-5 Transcription. The effect of cotreating EtOH-exposed cells with Rf effectively down-regulated the transcription of claudin-5 to the extent that the effects of EtOH were not presented or reversed (see Fig. 3*C*). The data clearly indicated that low concentrations of Rf have a powerful ability to suppress the transcription of claudin-5 and supersedes any concurrent effects of EtOH treatment.

DISCUSSION

Exposure of alcohol to the BBB is reported to increase the levels of reactive oxygen species in the BECs (Abdul Muneer et al., 2011; Haorah et al., 2005a, 2005b), resulting in a range of adverse effects, from affecting paracellular TJs and increasing permeability across the BBB to impaired glucose uptake by ECs (Abdul Muneer et al., 2011). In this study, we examined the effects of 2 selected doses of EtOH on the brain endothelial cell line (bEnd5). EtOH plasma concentrations of 10 to 40 mM have been reported to ensue following moderate EtOH consumption (Elamin et al., 2012), while plasma concentrations of 100 mM EtOH can be interpreted as a supraphysiological dose of EtOH, as the latter concentration of alcohol is rarely found in plasma with the exception of some extreme and excessive binge drinking (Sommer and Spanagel, 2013).

Furthermore, we tested the hypothesis that the simultaneous treatment with Rf, an established tincture known for its

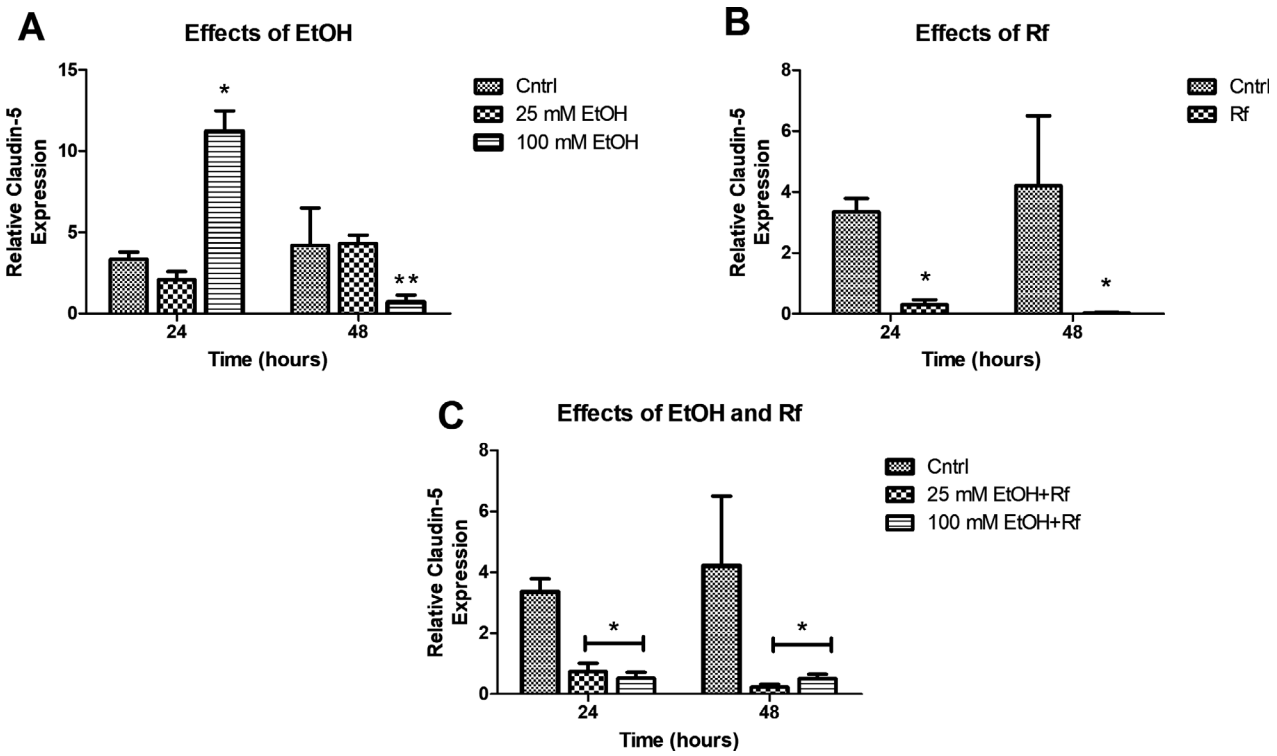

Fig. 3. Effects of EtOH and Rf on transcription. (**A**) The effects of 25 and 100 mM EtOH following 24-hour exposure on the relative mRNA transcription of claudin-5 after normalization with HPRT and GAPDH. *$p < 0.05$ designates a statistical decrease compared to controls, while $^{\#}p < 0.05$ designates a statistical increase compared to controls ($n = 6$). (**B**) The effects of Rf following 24-hour exposure on the relative mRNA transcription of claudin-5 after normalization with HPRT and GAPDH ($n = 6$). (**C**) The effects of combinations of EtOH and Rf following 24-hour exposure on the relative mRNA transcription of claudin-5 after normalization with HPRT and GAPDH. *$p < 0.05$ designates statistical significance compared to controls analyzed using the Mann–Whitney test. Results were displayed as mean ± SEM ($n = 6$).

high levels of AOs, would result in the amelioration of the negative effects of alcohol.

We previously reported on the detrimental effects of high concentrations of AOs (reductive stress) on the BECs (Mentor and Fisher, 2017), and in this study, to avoid the complicating effects of excessive AOs and its associated reductive stress, we approximated as closely as possible the effects of drinking a cup of Rf tea (Rooibos tea). This was in alignment with Breiter et al. (2011) who reported that following the consumption of 500 ml of Rf, the peak plasma concentrations of the individuals contained approximately 1.9 nM Aspalathin. Aspalathin represents overwhelmingly the main AO in the Rf tincture, hence the name, *Aspalathin linearis* (see Mentor and Fisher (2017) for the molecular analysis of Rf). We therefore added 0.08% Rf to our culture media, which equated to an equivalence of 1.9 nM Aspalathin in culture media. It was essential in evaluating the physiological effects on cultured cells that we used Aspalathin concentrations that were closely aligned to reported plasma concentrations, especially since the media bathing cultured BECs is analogous to the plasma of blood. Furthermore, the value of using a concentration of Rf that closely approximates the level of the bioactives of Rf in the plasma, following the consumption of 500 ml of the tincture, is that for the first time, we have been able to gauge the physiological effects of consuming AOs contained in teas. Most other studies (Kawano et al., 2009; Marnewick et al., 2011; Opuwari and Monsees, 2014; Waisundara and Hoon, 2015) have grossly overestimated the concentrations of Rf to be used in both *in vivo* studies and *in vitro* cell culture studies, as individuals would have to consume liters of the tincture to obtain similar plasma concentrations. Thus, throughout this study we treated the cells with Rf concentrations equivalent to plasma levels of Aspalathin found in patients who were exposed to 500 ml of Rf tea, which meant that cells were exposed to a miniscule 0.08% of Rf.

Endothelial Cell Viability

Given the low concentration of Rf, it was therefore not surprising that the viability (toxicity) of cells treated with Rf was not statistically different from control (see Fig. 1*E*). However, both concentrations of EtOH also had no statistical effect on the viability of the ECs (Fig. 1*E-F*). Cotreatment of BECs with Rf and EtOH at both doses had no statistical effects on the viability, except for 100 mM at 24 hours, where the viability % was recorded at 94.6%. Although this was statistically different from control ($p < 0.05$), it fell well within what is considered the normal range of viability for untreated cells (85 to 98%) and thus

cannot be construed to have any physiological significance. This was also expected as EtOH is proposed to bring about its effects by increasing ROS (Haorah et al., 2005a, 2005b), and cotreating the cells with Rf, rich in AOs, would have been able to nullify the ensuing OS (Fig. 1*E-F*). These results confirm our hypothetical position that the decrease in cell numbers was indeed not due to the toxicity of the EtOH treatment.

Cellular Proliferation

Effects of EtOH. Our experiments showed that exposure of cultured bEnd5 cells to EtOH (25 and 100 mM) for 24 hours significantly suppressed cellular proliferation. Our data were endorsed by the work of Mikami et al (1997) who reported that exposure to various concentrations of EtOH (12.5, 50, 100, and 200 mM) on a fibroblastic cell line derived from mouse connective tissue resulted in the suppression of cell division, induced by a transient G2/M block, inhibiting mitosis [but also reported that with longer exposure to EtOH, cells tended to acquire some tolerance enabling mitosis to proceed]. Our toxicology data (Fig. 1*B*) revealed the decrease in live cell numbers (proliferation) was not due to an increase in dead cells, further endorsing that bEnd5 cell division was suppressed. Haorah et al. (2005a, 2005b) linked BBB dysfunction to alcohol-induced OS, and Fisher et al (2015) showed that methamphetamine-induced OS was linked to the suppression in bEnd5 cell division, transiently blocking mitosis by preventing cells from entering into the S-phase of mitosis. It is, therefore, highly probable that the suppression of cell division was due to an alcohol-induced increase in ROS, leading to the impairment of the mitotic cell cycle.

Effects of Rf. The effects of Rf on live cell numbers over 48 hours were not surprising, not only that the dose was based on very low postabsorptive plasma concentrations (0.08% $\equiv$ 1.9 nM Aspalathin), but that the low dose of Rf negates the plausibility that Rf-derived AOs may have induced reductive stress. Many studies have pointed to the importance of normal cellular ROS production and its induction of endogenous AO systems (GSH, catalase, and SOD) in triggering cellular division (Naregal et al., 2017).

Cotreatment of EtOH and Rf. The suppressive effects of alcohol on cell division are based on the premise that firstly, the BEC's endogenous AO concentrations are not sufficient to neutralize excess ROS, and secondly, the cells' genomic response to insufficient endogenous AOs is not timeous to produce additional AOs. Thus, based on the premise that the negative effects of alcohol are predominantly brought about by inducing excess ROS, which exerts OS in the ECs, which in turn suppresses division, we examined the effects of adding exogenous AOs (Rf) to address this shortfall in cellular AOs.

We found that with the combinatorial exposure of EtOH and Rf, the effects of 25 mM EtOH on the number of live cells across the experimental time frame of 48 hours were fully reversed. Simultaneous exposure to Rf and 100 mM EtOH also fully reversed the effects of 100 mM EtOH on the rate of cell division in bEnd5 cells. Therefore, our data support our hypothesis that the alcohol-induced suppression of cell division via OS can be reversed by the addition of low concentrations of exogenous AOs.

Effects of EtOH and Rf on Endothelial Permeability

It is expedient to discuss the data on the genomic expression of claudin-5 together with the TEER data to attempt to elucidate the underlying mechanisms affecting the physiological changes brought about by EtOH and Rf. Several studies have reported that the mechanism whereby alcohol affects the BBB permeability is through the disruption of the endothelial TJ augmenting BBB permeability (Abdul Muneer et al., 2011; Haorah et al., 2005a, 2005b; Singh et al., 2007). Our data support these findings showing that both 25 and 100 mM EtOH increased the permeability (increase TEER) across the bEnd5 cellular monolayer.

On the other hand, 25 mM EtOH did not statistically affect claudin-5 expression and yet transendothelial permeability was increased (TEER decreased). This indicated that effects of EtOH were downstream, compromising TJ protein functionality at the level of the paracellular membrane. This line of thinking was endorsed by the effects of 100 mM EtOH, which in spite of a 4-fold increase (24 hours) in the expression of claudin-5 still presented an increase in transendothelial permeability (decrease in TEER; Fig. 2*A*, *C*). Although increased claudin-5 expression does not necessitate the eventuality that TJ proteins will be incorporated in the paracellular membranes, suppressed expression of claudin-5 (Fig. 3*B*,*C*) always resulted in an increase in permeability (decrease in TEER: Fig. 2). Thus, control levels of TJ protein expression are a prerequisite for establishing normal permeability across endothelia.

In support of this view, Haorah et al. (2005a, 2005b) reported that EtOH-induced OS in brain microvascular ECs resulted in a 26% decrease in TEER through the disorganization of the cytoskeleton and TJ protein assembly. They also showed that the increase in permeability resulted in increased leukocyte migration across their model of the BBB. Abdul Muneer et al (2011) further showed that alcohol suppressed the expression of both TJ occludin and claudin-5 in mouse brain microvessels.

The effects of the 100 mM concentration on TEER at 48 hours should be seen in the context of its effects on tight junction expression of claudin-5. At 48 hours, the expression of claudin-5 suppressed by 100 mM EtOH, thus leading to a compromise in the TJ sealing of the paracellular spaces between adjacent Bend5 cells. This resulted in an increase in transepithelial permeability (reflected by a decrease in TEER). Although the expression of claudin-5 at 24 hours is overexpressed, permeability across the Bend5 cell monolayer increases (decreased TEER) and this should be seen in the

context that 100 mM EtOH must be having its effects downstream of the expression of claudin-5, because although claudin-5 expression was not suppressed for the 25 mM EtOH-treated cells, TEER was significantly reduced at both 24 and 48 hours. Thus, if TEER is suppressed while claudin-5 TJs are expressed, it infers that EtOH brings about its effects by compromising TJ protein functionality at the paracellular membrane/space. The literature supports this view, as EtOH induces its effects via increase in ROS production, and increased ROS is implicated in interfering with scaffolding protein organization, with the phosphorylation of TJ proteins, as well as with TJ protein expression (Haorah et al., 2005a, 2005b). Thus, although TJ expression is crucial for TJ protein formation, and therefore for transepithelial impermeability, an increase in the expression of TJ protein expression does not guarantee its functional role at the paracellular membrane interface of adjacent endothelial brain cells.

The Effects of Rf on Endothelial Permeability. Under normal culture conditions, BECs are in redox balance (laboratory data). However, treatment with Rf caused a decrease in the expression of claudin-5 at both 24 and 48 hours ($p < 0.01$). This was reflected by an increase in transendothelial permeability (decrease in TEER) across the experimental time frame of 96 hours (Fig. 2*B*). Thus, the mechanism whereby Rf brought about its effects on transendothelial permeability was fundamental by suppressing claudin-5 expression.

Furthermore, based on our viability data (Fig. 1*E*), where Rf had no statistical effect, we were surprised that exposed monolayers of bEnd5 ECs to low concentrations of exogenous AOs (Rf) affected the expression of claudin-5. These data point to the sensitivity of the physiological redox balance of BECs (Poljsak et al., 2013). An alternative explanation is that the low concentration of AOs in the treatment protocol suggests that the mechanism causing the increase in permeability is not linked to redox balance in these BECs, but rather that some other molecule in the Rf tincture is responsible for this effect. This interesting effect is of interest as it may provide an avenue for the *ad hoc* regulation of the permeability of BBB and requires more research.

EtOH and Rf on Endothelial Permeability. Our hypothesis was that the Rf-derived AOs would neutralize EtOH-induced ROS, effectively reversing or nullifying the effects of EtOH-induced permeability across the bEnd5 brain endothelial monolayers. However, cotreatment of Bend5 monolayers with EtOH and RF could not reverse the EtOH-induced increase in transendothelial permeability. This was not as surprising given that the treatment of Rf suppresses the expression of claudin-5. This view is supported by the cotreated TEER data, where cells exposed to both EtOH and Rf presented suppressed claudin-5 expression and also brought about increased transendothelial permeability (decreased TEER). In addition, although 25 mM EtOH independently had no statistical effect on claudin-5 expression, and at 24 hours, 100 mM had caused a 4-fold increase in claudin-5 expression, cotreatment with Rf caused decreased TEER and suppressed claudin-5 expression. This was indicative of the suppressive effects of Rf on the expression of claudin-5.

The increase in TEER at 72 and 96 hours may represent a partial recovery. In view that cells were only treated for 24 hours with EtOH, this partial recovery in TEER is seen both with cells treated only with EtOH (Fig. 2*A*) and when cells were cotreated with EtOH and RF (Fig. 2*C*).

These data are endorsed in the literature where EtOH has been linked to an increase in permeability across BEC monolayers through the compromise of paracellular TJs (Abdul Muneer et al., 2011; Haorah et al., 2005a, 2005b; Singh et al., 2007). Abdul Muneer et al (2011) showed that occludin, claudin-5, and ZO-1 expression was significantly suppressed by EtOH exposure. Furthermore, the integrity of TJs is intimately linked to the actin cytoskeleton via intracellular proteins zonula occludens (ZO-1-3). Haorah et al. (2008a, 2008b) showed that BECs after exposure to EtOH resulted in the phosphorylation of TJ proteins, which control TJ assembly and BBB integrity. It is clear that the regulation of TJs is multifaceted, and many signaling pathways are responsible for the integrity of paracellular permeability (Li et al., 2012). In view of the multifaceted nature of paracellular TJ regulation, it is plausible that EtOH and its metabolites in BECs affect different pathways to that of Rf and its main AO, Aspalathin.

TJ Expression

In this study, we found that treated and untreated bEnd5 cells did not express the TJ protein occludin. Steiner et al (2011) also reported that bEnd5 cells lacked the localization of occludin to cellular junctions; however, Findley and Koval (2009) established that occludin-deficient mice are viable and exhibit normal barrier function. Furthermore, the bEnd5 cells used in this study have successfully expressed one of the critical proteins of brain endothelial cell TJs, claudin-5 (Fisher and Mentor, 2020). In BECs, claudin-5 mRNA is almost 600-fold greater than claudin-3 (Günzel and Yu, 2013). Furthermore, the importance of claudin-5 TJ proteins to the brain function is illustrated in that claudin-5-knockout mice never survive for more than 10 hours after birth (Fisher and Mentor, 2020).

EtOH on Claudin-5 Expression. The transcription of claudin-5 in BECs exposed to 25 mM EtOH changed dramatically across the 48-hour time frame. In addition, as reported, the transcription of claudin-5 in response to the 25 mM concentration of EtOH was not statistically affected between 24 and 48 hours. Cells exposed to 100 mM EtOH induced an irregular trend in claudin-5 transcription, with a 4-fold increase in transcription at 24 hours. The daily variability in both control claudin-5 levels, and those of the EtOH-treated BECs, suggests that transcription of claudin-5 is regulated by particularly dynamic processes, which may

vary on a daily/hourly basis. Schramm et al. (2017) showed that decreasing calcium concentration resulted in the rapid disassembly of TJs (within 20 minutes) causing a sharp decrease in TEER. In support of this view, Abdul Muneer et al (2011) established in a study that EtOH suppressed the transcription of TJ proteins, occludin, claudin-5, and ZO-1, thereby altering TJ assembly, and ultimately impairing BBB integrity, which is coherent with the suppression in claudin-5 transcription at 48 hours in our study. Furthermore, it is plausible that the toxic metabolite of EtOH, acetyl acetate, also strongly contributes to the modifications in the molecular composition of TJs and its associated cytoplasmic molecules (Elamin et al., 2012).

Rf on Claudin-5 Expression. During the investigation of the effects of Rf on claudin-5 transcription, we established that Rf resulted in an overall down-regulation of claudin-5 throughout the course of the experiment. To the best of our knowledge, the effects of Rf on claudin-5 transcription have never before been reported in the bEnd5 cell line. In support of our data, green tea polyphenols (a tea containing high concentrations of AOs) were reported to also decrease claudin-5 expression similarly to what was observed in our study (Liu et al., 2013). The data strongly indicate that the addition of AOs to cultured cells may disturb the intracellular redox equilibrium, compromising the assembly of TJ proteins in the paracellular pathway of the BBB. Furthermore, the comparison of the continuous suppression of claudin-5 in Rf-exposed cells compared to the pattern-less effects of EtOH also points to different cellular mechanisms. These data strongly endorse our permeability study, which shows that Rf decreased TEER (increased permeability) throughout the experimental time frame. The data show that down-regulation of the primary TJ protein, claudin-5, leads to increased paracellular permeability.

EtOH and Rf on Claudin-5 Expression. The cotreatment of Rf and EtOH resulted in suppression of claudin-5 throughout the 48-hour time frame of the experiment. As discussed above, it is not clear as to the exact mechanism used by Rf and EtOH to suppress the transcription of the TJ proteins. Further complicating the understanding of the mechanism of action is the level at which EtOH and Rf bring about their suppression of TJ proteins: Interference could be via one of the signaling of genomic pathways designed to trigger transcription of TJ protein mRNA (within the nucleus), or it could be at level of the translational step (cytoplasmic), or/and it could be at the posttranslation phase of the expression of the TJ proteins. Our PCR data indicate that cotreatment of Rf/EtOH appeared to reinforce the suppressive effects on TJ mRNA expression and, furthermore, it must be pointed out that Rf suppressed the variability in claudin-5 expression observed during the EtOH-only treatment of the BECs. These concurrent effects infer that a common redox mechanism exists, which affects paracellular TJ expression and which is particularly sensitive to exogenous AOs, and that further research is required to elucidate and clarify the exact mechanisms.

The suppression of the claudin-5 TJ proteins by both EtOH and Rf leads to an increase in permeability via the paracellular pathway. Our TEER data, strongly supported the PCR data, endorsed that increased transendothelial permeability was caused predominantly by compromised TJ proteins in the paracellular pathways across monolayers layers of BECs, and may not involve the transcellular pathways.

CONCLUSION

A key observation in this study was that Rf was able to reverse the negative effects of EtOH-induced suppression of cell division, however, it was not able to reverse the effects of EtOH on the permeability across monolayers of BECs (bEnd5), nor reduce the effects of EtOH in suppressing the expression of claudin-5, a TJ protein which is crucial in establishing paracellular impermeability.

A second key observation was that the very low plasma concentrations of Rf, with an equivalence of 1.9 nM Aspalathin, are able to physiologically impact crucial regulatory elements of the BBB's ECs, effecting both endothelial cell division and also BBB permeability.

Our study shows for the first time the low concentrations of AOs have the ability to significantly increase BEC permeability and suppress expression of the paracellular TJ protein claudin-5. Our study also confirms the established relationship between the suppression of claudin-5 expression and increase in transendothelial permeability (decrease in TEER) is indicative of compromised TJ protein functionality and increased paracellular permeability. It is clear from the data in this study that the effects of introducing exogenous AOs have physiological implications for cellular systems, which requires additional introspection. This study suggests that even low concentrations of exogenous AOs could compromise the redox equilibrium of BECs affecting its ability to regulate brain homeostasis.

ACKNOWLEDGMENTS

This work was supported by the University of the Western Cape and the Neurobiology Group in the Department of Medical Biosciences. A 20% Rf extract was profiled at the Oxidative Stress Research Centre (Mentor and Fisher, 2017), at the Cape Peninsula University of Technology (CPUT), and we are grateful to Mr. F. Rautenbach for performing the chemical and HPLC analysis of an aqueous extract of Rf. The authors are grateful to the National Research Foundation and the Senate Research Committee (UWC) for funding this project.

CONFLICT OF INTEREST

All contributing authors declare that they have no conflict of interest.

REFERENCES

Abdul Muneer PM, Alikunju S, Szlachetka AM, Haorah J (2011) Inhibitory effects of alcohol on glucose transport across the blood-brain barrier leads to neurodegeneration: preventive role of acetyl-L-carnitine. Psychopharmacology 214:707–718.

Berker KI, Olgun FAO, Ozyurt D, Demirata B, Apak R (2013) Modified Folin-Ciocalteu antioxidant capacity assay for measuring lipophilic antioxidants. J Agric Food Chem 61:4783–4791.

Breiter T, Laue C, Kressel G, Gröll S, Engelhardt UH, Hahn A (2011) Bioavailability and antioxidant potential of rooibos flavonoids in humans following the consumption of different rooibos formulations. Food Chem 128:338–347.

Colgan OC, Collins NT, Ferguson G, Murphy RP, Birney YA, Cahill PA, Cummins PM (2008) Influence of basolateral condition on the regulation of brain microvascular endothelial tight junction properties and barrier function. Brain Res 1193:84–92.

Dai J, Mumper RJ (2010) Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. Molecules 15:7313–7352.

Derk R, Davidson DC, Manke A, Stueckle TA, Rojanasakul Y, Wang L (2015) Potential *in vitro* model for testing the effect of exposure to nanoparticles on the lung alveolar epithelial barrier. Sens Bio-sens Res 3:38–45.

Elamin E, Jonkers D, Juuti-Uusitalo K, Van Ijzendoorn S, Troost F, Duimel H, Broers J, Verheyen F, Dekker J, Masclee A (2012) Effects of ethanol and acetaldehyde on tight junction integrity: in vitro study in a three dimensional intestinal epithelial cell culture model. PLoS One 7:e35008.

Findley MK, Koval M (2009) Regulation and roles for claudin-family tight junction proteins. IUBMB Life 61:431–437.

Fisher D, Gamieldien K, Mafunda PS (2015) Methamphetamine is not toxic but disrupts the cell cycle of the blood brain barrier endothelial cells. Neurotox Res 28:8–17.

Fisher D, Mentor S (2020) Are claudin-5 tight-junction proteins in the blood-brain barrier porous? Neural Regen Res 15:1838–1839.

Gabathuler R (2010) Approaches to transport therapeutic drugs across the blood-brain barrier to treat brain diseases. Neurobiol Dis 37:48–57.

Günzel D, Yu ASL (2013) Claudins and the modulation of tight junction permeability. Physiol Rev 93:525–569.

Haorah J, Heilman D, Knipe B, Chrastil J, Leibhart J, Ghorpade A, Miller DW, Persidsky Y (2005) Ethanol-induced activation of myosin light chain kinase leads to dysfunction of tight junctions and blood-brain barrier compromise. Alcohol Clin Exp Res 29:999–1009.

Haorah J, Knipe B, Leibhart J, Ghorpade A, Persidsky Y (2005) Alcohol-induced oxidative stress in brain endothelial cells causes blood-brain barrier dysfunction. J Leukoc Biol 78:1223–1232.

Haorah J, Ramirez SH, Floreani N, Gorantla S, Morsey B, Persidsky Y (2008) Mechanism of alcohol-induced oxidative stress and neuronal injury. Free Radic Biol Med 45:1542–1550.

Haorah J, Schall K, Ramirez SH, Persidsky Y (2008) Activation of protein tyrosine kinases and matrix metalloproteinases causes blood-brain barrier injury: novel mechanism for neurodegeneration associated with alcohol abuse. Glia 56:78–88.

Huang MC, Chen CC, Peng FC, Tang SH, Chen CH (2009) The correlation between early alcohol withdrawal severity and oxidative stress in patients with alcohol dependence. Prog Neuro-Psychopharmacol Biol 33:66–69.

Joubert E, Schulz H (2006) Production and quality aspects of rooibos tea and related products. A review. J App Bot Food Qual 80:138–144.

Kawano A, Nakamura H, Hata S, Minakawa M, Miura Y, Yagasaki K (2009) Hypoglycemic effect of aspalathin, a rooibos tea component from *Aspalathus linearis*, in type 2 diabetic model db/db mice. Phytomedicine 16:437–443.

Krafczyk N, Glomb MA (2008) Characterization of phenolic compounds in rooibos tea. J Agric Food Chem 56:3368–3376.

Li J, Ye L, Wang X, Liu J, Wang Y, Zhou Y, Ho W (2012) (−)-Epigallocatechin gallate inhibits endotoxin-induced expression of inflammatory cytokines in human cerebral microvascular endothelial cells. J Neuroinflamm 9:161–174.

Liu X, Wang Z, Wang P, Yu B, Liu Y, Xue Y (2013) Green tea polyphenols alleviate early BBB damage during experimental focal cerebral ischemia through regulating tight junctions and PKC alpha signaling. BMC Complement Altern Med 13:187–197.

Mandyam CD, Villalpando EG, Steiner NL, Quach LW, Fannon MJ, Somkuwar SS (2017) Platelet endothelial cell adhesion molecule-1 and oligodendrogenesis: significance of alcohol use disorders. Brain Sci 7:131–149.

Marnewick JL, Rautenbach F, Venter I, Neethling H, Blackhurst DM, Wolmarans P, Macharia M (2011) Effects of rooibos (*Aspalathus linearis*) on oxidative stress and biochemical parameters in adults at risk for cardiovascular disease. J Ethnopharmacol 133:46–52.

Mentor S, Fisher D (2017) Aggressive antioxidant reductive stress impairs brain endothelial cell angiogenesis and blood brain barrier function. Curr Neurovasc Res 14:71–81.

Mikami K, Haseba T, Ohno Y (1997) Ethanol induces transient arrest of cell division (G2 + M block) followed by G0/G1 block: dose effects of short- and longer-term ethanol exposure on cell cycle and cell functions. Alcohol 32:145–152.

Naregal GV, Devaranavadagi BB, Patil SG, Aski BS (2017) Elevation of oxidative stress and decline in endogenous antioxidant defense in elderly individuals with hypertension. J Clin Diagn Res 11:BC09–BC12.

Opuwari CS, Monsees TK (2014) In vivo effects of *Aspalathus linearis* (rooibos) on male rat reproductive functions. Andrologia 46:867–877.

Pardridge WM (1995) Transport of small molecules through the blood–brain barrier: biology and methodology. Adv Drug Deliv 15:5–36.

Pengilly M, Joubert E, Van Zyl WH, Botha A, Bloom M (2008) Enhancement of Rooibos (*Aspalathus linearis*) aqueous extract and antioxidant yield with fungal enzymes. J Agric Food Chem 56:4057–4053.

Poljsak B, Suput D, Milisav I (2013) Achieving the balance between ROS and antioxidants: when to use the synthetic antioxidants. Oxid Med Cell Longev 2013:956792.

Schramm RA, Koslow MH, Nelson DA, Larsen M, Castracane J (2017) A novel impedance biosensor for measurement of trans-epithelial resistance in cells cultured on nanofiber scaffolds. Biosensors 7:35–45.

Schreibelt G, Kooij G, Reijerkerk A, van Doorn R, Gringhuis SI, van der Pol S, Weksler BB, Romero IA, Couraud PO, Piontek J, Blasig IE, Dijkstra CD, Ronken E, de Vries HE (2007) Reactive oxygen species alter brain endothelial tight junction dynamics via RhoA, PI3 kinase, and PKB signaling. FASEB J 21:3666–3676.

Singh AK, Jiang Y, Gupta S, Benlhabib E (2007) Effects of chronic ethanol drinking on the blood-brain barrier and ensuing neuronal toxicity in alcohol-preferring rats subjected to intraperitoneal LPS injection. Alcohol Alcohol 42:385–399.

Snijman PW, Joubert E, Ferreira D, Li X, Ding Y, Green IR, Gelderblom WCA (2009) Antioxidant activity of the dihydrochalcones aspalathin and nothofagin and their corresponding flavones in relation to other rooibos (*Aspalathus linearis*) flavonoids, epigallocatechin gallate, and trolox. J Agric Food Chem 57:6678–6684.

Sommer W, Spanagel R (2013) Behavioral neurobiology of alcohol addiction. Preface. Curr Top Behav Neurosci 13:v–vii.

Standley L, Winterton P, Marnewick JL, Gelderblom WCA, Joubert E, Britz TJ (2001) Influences of processing stages on antimutagenic and antioxidant potentials of rooibos tea. J Agric Food Chem 49:114–117.

Steiner O, Coisne C, Engelhardt B, Lyck R (2011) Comparison of immortalized bEnd5 and primary mouse brain microvascular endothelial cells as in vitro blood-brain barrier models for the study of T cell extravasation. J Cereb Blood Flow Metab 31:315–327.

Waisundara VY, Hoon LY (2015) Free radical scavenging ability of *Aspalathus linearis* in two in vitro models of diabetes and cancer. J Tradit Complement Med 5:174–178.

Wu D, Cederbaum AI (2003) Alcohol, oxidative stress, and free radical damage. Alcohol Res Health 27:277–284.