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Vitis labrusca extract effects on cellular dynamics and redox modulations in a SH-SY5Y neuronal cell model: A similar role to lithium

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ABSTRACT

Oxidative stress and calcium imbalance are consistently reported in bipolar disorder (BD). Polymorphism of voltage-dependent calcium channel, L type, alpha 1C subunit (CACNA1c), which is responsible for the regulation of calcium influx, was also shown to have a strong association with BD. These alterations can lead to a number of different consequences in the cell including production of reactive species causing oxidative damage to proteins, lipids and DNA. Lithium is the most frequent medication used for the treatment of BD. Despite lithium's effects, long-term use can result in many negative side effects. Therefore, there is an urgent need for the development of drugs that may have similar biological effects as lithium without the negative consequences. Moreover, polyphenols are secondary metabolites of plants that present multi-faceted molecular abilities, such as regulation of cellular responses. *Vitis labrusca* extract (VLE), a complex mixture of polyphenols obtained from seeds of winery wastes of *V. labrusca*, was previously characterized by our group. This extract presented powerful antioxidant and neuroprotective properties. Therefore, the ability of VLE to ameliorate the consequences of hydrogen peroxide (H_2O_2)-induced redox alterations to cell viability, intracellular calcium levels and the relative levels of the calcium channel CACNA1c in comparison to lithium's effects were evaluated using a neuroblastoma cell model. H_2O_2 treatment increased cell mortality through apoptotic and necrotic pathways leading to an increase in intracellular calcium levels and alterations to relative CACNA1c levels. VLE and lithium were found to similarly ameliorate cell mortality through regulation of the apoptotic/necrotic pathways, decreasing intracellular calcium levels and preventing alterations to the relative levels of CACNA1c. The findings of this study suggest that VLE exhibits protective properties against oxidative stress-induced alterations similar to that of lithium. These findings suggest that VLE may be an attractive potential candidate as a novel therapeutic agent for BD.

1. Introduction

The pathophysiology of bipolar disorder (BD) is related to a number of factors that include genetic causes, neurotransmitter dysregulation and oxidative stress (Salvadore et al., 2010). In particular, genome-wide association studies (GWAS) have revealed a strong association between polymorphism of the CACNA1c (voltage-dependent calcium channel, L type, alpha 1C subunit) gene and BD (Ferreira et al., 2008; Green et al., 2010), bringing attention to the

Abbreviations: AMPK, AMP-activated protein kinase; Annexin V+, annexin V positive cells; ATP, adenosine triphosphate; BBB, blood brain barrier; Bcl-2, B-cell CLL/lymphoma 2; BD, bipolar disorder; BDNF, brain derived neurotrophic factor; CACNA1c, voltage-dependent calcium channel, L type, alpha 1C subunit; DAPI, 4′,6-diamidino-2-phenylindole; DNA, deoxyribonucleic acid; DMSO, dimethyl sulfoxide; DPBS, Dulbecco's Phosphate-Buffered Saline; FBS, fetal bovine serum; Fluo-4AM, non-fluorescent acetoxymethyl ester; Fura-Red AM, green-fluorescent acetoxymethyl ester; GSK3β, glycogen synthase kinase 3 beta; GWAS, genome-wide association studies; Hep G2, human hepatocellular carcinoma; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; OIF, original imaging format; PARP, poly (ADP-ribose) polymerase; PI, propidium iodide; RNS, reactive nitrogen species; ROI, regions of interest; ROS, reactive oxygen species; RT, room temperature; RT-PCR, real-time polymerase chain reaction; SH-SY5Y, human subline of the neuroblastoma cell line SK-N-SH; VLE, *Vitis labrusca* extract.

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role that calcium imbalance may play in BD. Redox modulations associated with mitochondrial dysfunction through alterations of the electron transport chain are also observed in BD (Scola et al., 2013b). This deregulation can lead to a number of different consequences including production of reactive oxygen and nitrogen species (ROS and RNS) and thereafter oxidative insults to proteins, lipids and DNA (Andreazza et al., 2008; Halliwell, 1992). Indeed, oxidative damage has been found in patients with BD (Andreazza, 2012; Clay et al., 2010; Konradi et al., 2012). Lithium is the most frequently used medication for the treatment of BD (Kupfer, 2005). Several hypotheses have been postulated to explain lithium's mood-stabilizing ability in BD, such as improving the mitochondrial electron transport chain (Andreazza et al., 2010; Maurer et al., 2009) and protecting macromolecules against redox modifications (Frey et al., 2006; Jornada et al., 2011; Khairova et al., 2012; Soeiro-de-Souza et al., 2013). Lithium is known to operate on a number of pathways, including activation of the Wnt signaling pathway, inhibition of glycogen synthase kinase 3β (GSK-3β) and regulation of calcium levels in patients with BD to list a few (Boyadjieva and Varadinova, 2012; Malhi et al., 2013). Despite lithium's abilities, long-term use can result in many negative side effects including hypothyroidism (Kleiner et al., 1999), decline in renal function (Markowitz et al., 2000) and memory disturbance (Young and Newham, 2006). Therefore, there is a pressing need for the development of drugs with similar biological effects as lithium, including amelioration of redox modifications, without its harmful side effects.

A potential candidate previously characterized by our group is a winery waste grape seed extract, *Vitis labrusca* extract (VLE). Some studies have been conducted to explore the antioxidant and neuroprotective properties of *V. labrusca* (Cardozo et al., 2013; Dani et al., 2010; Rodrigues et al., 2012; Scola et al., 2010, 2013a, 2013c), therefore its unique qualities and capabilities are not yet well known and further research is required to examine its therapeutic possibilities. VLE is a polyphenolic complex mixture obtained from the grape seeds containing 134 mg/L of catechin, 130 mg/L of epicatechin, 17 mg/L of procyanidin B1, 15 mg/L of procyanidin B2, 13 mg/L of procyanidin B3, 7 mg/L of epigallocatechin and 2 mg/L of procyanidin B4 (Scola et al., 2010), which present neuroprotective effects as previously found by Scola et al. (2013c). Polyphenols from the grape seed, known as flavan-3-ol, are capable of crossing the blood brain barrier (BBB) (Faria et al., 2011; Schaffer and Halliwell, 2012) and have been shown to increase cerebral blood flow (Schaffer and Halliwell, 2012). The bioavailability of polyphenols from grape seed can be enhanced, in brain and plasma, by repeated administration (Ferruzzi et al., 2009). For more details on bioavailability of grape seed compounds and its metabolites refer to Ferruzzi et al., 2009.

Grape seed polyphenols are multi-faceted molecules with strong antioxidant abilities that were shown to promote hippocampal neuroprotection (Narita et al., 2011), improve cellular growth of mouse epithelial cells (Takahashi et al., 1999) and modulate inflammatory response in astrocytes (Fujishita et al., 2009). Importantly, they were also shown not to be genotoxic in rat lymphocytes nor mutagenic in XV185-14c *Saccharomyces cerevisiae* (Scola et al., 2013c). Moreover, flavan-3-ols can induce an upregulation in antioxidant gene expression, as described by Niture et al. (2014) in Hep G2 cells, which could lead to an enhancement in endogenous antioxidant defenses.

As aforementioned, oxidative stress and calcium imbalance are central in the pathophysiology of BD and lithium's mechanism of action. Because polyphenols can regulate redox modifications and penetrate the BBB, they may be able to activate the same pathways as lithium without resulting in significant side effects, making them an attractive potential candidate as a novel therapeutic agent for BD. Hence, the objective of this study was to explore the ability of VLE to ameliorate the consequences of hydrogen peroxide (H_2O_2)-induced redox alterations. This includes cell viability, intracellular calcium levels and the relative levels of the calcium channel CACNA1c in comparison to lithium's effects using a neuroblastoma cell model. These novel findings may demonstrate VLE's potential to be used as an adjunct therapy for patients with BD.

2. Experimental procedures

2.1. VLE

V. labrusca (cv. Bordo) was cultivated in the northeast area of Rio Grande do Sul, Brazil and the voucher specimen was identified by the herbarium of the University of Caxias do Sul, Brazil (HUCS31065). Winery waste seeds of *V. labrusca* were removed from the vinification tanks 5 days after the beginning of fermentation in January 2011 and were extracted as 5 g of seeds/100 mL of distilled water, under reflux (100°C; 30 min), filtered through a 0.45 μm pore filter (Millipore) and freeze-dried. The extract was solubilized in Ham's F12 medium immediately before use. Some of the major compounds of VLE are catechin, epicatechin, epigallocatechin, procyanidins (B1, B2, B3, B4) (as described in Scola et al., 2010) (Scola et al., 2010). VLE is freely available to colleagues in academic research upon request.

2.2. Cell culture

Neuroblastoma cell line SH-SY5Y (ATCC CRL-266, VA, US) was cultured in Ham's F12 medium supplemented with 10% fetal bovine serum (FBS; Invitrogen; Canadian origin) and 1% penicillin/streptomycin (Gibco) and maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C for 3–6 days before experimentation. Cells were treated with VLE (50 ng/mL) or lithium (0.75 mM) for 72 hours and treated with different concentrations of hydrogen peroxide (H_2O_2; 5, 10, 25, 50 and 100 μM; Sigma, St. Louis, MO) for 30 minutes. After washout, cells were cultured for another 24 hours.

2.3. Cell viability

CellTiter-Blue® Cell Viability Assay (Promega, US) was used to determine cell viability. Cells (4×10^4 per well in 96-well plate) were treated with VLE (50 ng/mL) or lithium (0.75 mM) for 72 hours, and treated with different concentrations of H_2O_2 (5, 10, 25, 50 and 100 μM). Cells were grown in media without FBS for 24 hours and assayed for viability according to manufacturer's instructions. Data are presented as percent of non-VLE or lithium-treated control (N = 8).

2.4. Annexin V and propidium iodide assay and image analysis

To estimate the different levels of apoptotic and necrotic cells, the ApoDETECT Annexin V-FITC kit (Invitrogen) was used according to manufacturer's instructions. Annexin V labels phosphatidylserine that has been exposed during apoptosis and propidium iodide (PI) enters the cell when membrane integrity is lost during necrosis. Cells (2×10^5 cells per well in 24-well plate) were pretreated with media, VLE (50 ng/mL) or lithium (0.75 mM) for 72 hours followed by treatment with different concentrations of H_2O_2 (5, 10 and 50 μM). Cells were visualized using a confocal laser scanning microscope. The number of annexin V only positive cells, cells positive for PI, and total number of cells were counted by two examiners based on previously published methods (Farinacci, 2007; Schutte et al., 1998). Cells positive only for annexin V were considered apoptotic, cells positive for PI were considered necrotic (Weber et al., 2004). *Image Analysis:* images were taken using