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Intravenous nanosomes of quercetin improve brain function and hemodynamic instability after severe hypoxia in newborn piglets

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ABSTRACT

Perinatal asphyxia is a major cause of death and neurological morbidity in newborns and oxidative stress is one of the critical mechanisms leading to permanent brain lesions in this pathology. In this context we have chosen quercetin, a natural antioxidant, known also by its brain protective effects to study its potential as a therapy for brain pathology provoked by severe hypoxia in the brain. To overcame the difficulties of quercetin to access the brain, we have developed lecithin/cholesterol/cyclodextrin nanosomes as a safe and protective vehicle.

We have applied the nanosomal preparation intravenously to newborn piglets submitted to a severe hypoxic or ischemic/hypoxic episode and followed them for 8 or 72 h, respectively. Either towards the end of 8 h after hypoxia or up to 72 h after, electroencephalographic amplitude records in animals that received the nanosomes improved significantly. Animals receiving quercetin also stabilized blood pressure and recovered spontaneous breathing. In this experimental group mechanical ventilation assistance was withdrawn in the first 24 h while the hypoxic and vehicle groups required more than 24 h of mechanical ventilation. Three days after the hypoxia the suckling and walking capacity in the group that received quercetin recovered significantly compared with the hypoxic groups. Pathological studies did not show significant differences in the brain of newborn piglets treated with nanosomes compared with hypoxic groups. The beneficial effects of quercetin nanosomal preparation after experimental perinatal asphyxia show it as a promising putative treatment for the damaged brain in development.

1. Introduction

Perinatal asphyxia is a major cause of death and neurological morbidity in newborns. Estimates of the incidence of severe perinatal asphyxia (causing death or severe neurological impairment) is 1/1000 live births in industrial countries increasing to 5–10/1000 live births in low-income countries (McGuire, 2007). The severity of the asphyxia leads to hypoxic–ischemic encephalopathy (HIE), which remains a major cause of disability such as cerebral palsy, developmental delay, visual and hearing impairment and learning and behavioral problems. Early interventions to minimize brain damage are critical, and immediate resuscitation to restore oxygen supply and blood circulation aims to limit the extent of neuronal damage. Oxidative stress and high intracellular calcium leading to apoptosis are some of the mechanisms postulated to be important in this process (McGuire, 2007).

Despite intensive research, the therapeutic arsenal available to help those infants at high risk of neuropathological lesions is limited, and even induced hypothermia, a therapeutic tool widely utilized, shows limited beneficial outcomes in the long term; i.e., six or seven years after the asphyctic episode (Battin et al., 2001; Edwards and Azzopardi, 2006; Wintermark, 2011; Shankaran et al., 2012).

Although the brain is the most affected system, and neuropathological lesions can lead to sequelae, peripheral hemodynamic changes and multi-organ dysfunction can deepen the severity of the systemic pathology, sometimes becoming the cause of death. In this context, the selection of the best models mimicking human perinatal asphyxia is critical for understanding the physiopathological changes and for testing new therapies.

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The newborn piglet severe hypoxia model has been established in the last few years as the most appropriate model. The genetic and physiological proximity of piglets to humans, the similar ontogeny of the brain, the possibility of inducing a comparable neuropathological lesion and the advantages of the size of the animal that allows the use of the same technology as in infants in critical care units, have been the main reasons for the selection of this model (Dobbing and Sands, 1979; Foster et al., 2001; Bjorkman et al., 2006; Faa et al., 2012).

Multiple pharmacological approaches have been studied in perinatal asphyxia to minimize brain damage, with no significant results (McGuire, 2007). Although oxidative stress is recognized as a major event in the pathological process (Inder and Volpe, 2000), mainly after resuscitation maneuvers are applied (Faa et al., 2012; Solberg et al., 2012), the results with antioxidant therapy are inconclusive. The complex brain pathological process in perinatal asphyxia critically requires appropriate bioavailability of pharmacological agents at the neuronal interface and nanoscale delivery systems are being increasingly utilized for adequate and safe brain delivery of therapeutic compounds (Gandhi et al., 2010).

In the last few years, several epidemiological and experimental studies have pointed to a role of natural antioxidants as neuroprotective agents in neurodegenerative and vascular disorders (Rivera et al., 2008; Silva et al., 2008; Vauzour et al., 2008). Flavonoids, ubiquitous molecules in plants, fruits and beverages such as tea have been identified as the main responsible agents in the protective profile (Dajas et al., 2003a; Patil et al., 2003; Youdim et al., 2004). Thus, multiple types of evidence have demonstrated that quercetin, a flavonoid abundant in plants, fruits and vegetables, protects neurons in culture against oxidative insults as well as neurons in *in vivo* models of focal ischemia or trauma in rats (Ossola et al., 2009; Dajas, 2012; Dajas et al., 2013). The beneficial effects of quercetin as an antioxidant, anti-inflammatory and promoter of gene expression of survival proteins make it a potential therapeutic alternative for HIE. Nevertheless, the low water solubility of quercetin and its blood metabolic process cause poor brain bioavailability, a pharmacological challenge that requires the use of drug delivery systems to improve quercetin access to the brain and other organs in acute treatments (Rivera et al., 2004; Priprem et al., 2008; Ghosh et al., 2009). Thus, we have developed lecithin/cholesterol/2-hydroxypropyl-β-cyclodextrin (2HBCD) nanosomes of quercetin, a delivery system that in preliminary studies has shown assurance of very good and safe bioavailability.

The aim of the present study was to test the hemodynamic safety of the new formulation in newborn piglets submitted to hypoxia and assess its neuroprotective capacity.

2. Methods

Newborn piglets (*sus scrofa domestica*) up to 48 h from birth with a body weight of 1500 g to 2000 g were transported on the day of the experiment from the local farm, "La Familia" (Pando, Canelones, Uruguay), to the University Hospital (n = 36). After careful washing, they were moved to the experimental setting. The National Ethical Committee on Animal Experimental work approved the experimental protocol (file number 071140-000261-11, CHEA, Faculty of Medicine, approved 1/6/2011).

2.1. Animal preparation and monitoring

Anesthesia was induced with an intramuscular injection of ketamine (Inducmina®, 50 mg/kg), and general deep anesthesia was maintained using an intravenous mixture of midazolam (Icu vita®) 6 mg, fentanyl (Cristalia®) 100 μg and ketamine 100 mg in 100 ml of saline at a flow rate of 6 ml/h during the whole experiment.

Piglets were monitored by pulse oximetry (basal oxygen saturation: $SatO_2$ 95–100%) and electrocardiogram (ECG; basal HR–HR was 130–170 beats/min). The esophageal temperature was monitored to maintain it with a heating blanket between 38.5 and 39.5 °C. Prior to surgical preparation, all piglets received intravenous antibiotics (cefradine – Winpharm®- 100 mg/kg, gentamicin –Verifar®- 4 mg/kg). The anesthetic state was regularly evaluated throughout the experimental period, taking into account spontaneous movements, reactivity of pupils and spontaneous ventilation.

After a neck incision, the trachea was exposed, and an endotracheal tube (3 mm or 3.5-mm diameter) was introduced to connect the piglet to a ventilator (Sechrist IV 200), which began assisted mechanical ventilation.

Catheters were inserted into superior cava vein and femoral artery to maintain anesthesia, continuous monitoring of arterial blood pressure and extraction of blood samples respectively.

Additionally, a one-channel electroencephalogram (EEG) in the left and right parietal position was recorded (inter-electrode distance of 3 cm). Skin impedance was lowered by shaving and washing, and impedance of the surface electrodes was always <10 kΩ during the recordings. The amplitude of the EEG signal was integrated and recorded in an Olympic Cerebral Function Monitor (CFM) 6000 before, during and for 8 h after the experimental severe hypoxia.

2.2. Bioavailability assessment

2.2.1. Plasma samples

In newborn piglets, anesthetized and monitored as described above, 10 mg/kg of nanosomes of quercetin were administered intravenously for 60 min as an infusion. Blood samples were taken before nanosome administration, 30 min and 60 min after the beginning of the nanosomal infusion. Five animals were used in these experiments.

Samples were centrifuged and plasma was kept frozen at −20 °C until assessment of quercetin by HPLC.

At the moment of HPLC analysis, 100 μl of plasma were added to 350 μl of MetOH (with added BHT and EDTA to prevent oxidation) and 50 μl of PCA 0.1 M. Luteolin 0.75 μg/ml was used as an internal standard. The mixture was centrifuged for 3 min at 2.000 rpm, and 200 μl of the supernatant was diluted in 200 μl of bi-distilled water. After another step of centrifugation for 15 min at 15,000 rpm (4 °C), 50 μl of the supernatant was injected into the HPLC column.

2.2.2. Brain samples

After the beginning of quercetin administration, a skull window in the parietal region was opened, and a small sample of up to 400 mg of cortical tissue was taken at 0, 30 and 60 min. The samples were washed with saline solution and kept frozen at −20 °C up to the moment of assessment.

At the moment of analysis, biopsy samples were thawed and mixed with 1400 μl of MetOH (with BHT and EDTA), 200 μl of PCA 0.1 M and 100 μl of luteolin (700 ng/ml). The mixture was sonicated and shaken for 10 min. After that, it was centrifuged for 3 min at 2000 rpm, and 400 μl of the supernatant plus 1600 μl of bidistilled water were passed through an extraction SPE column and the eluted materials were dried in a Speed Vac at 45 °C for 3 h. The tubes containing the dried samples were supplemented with 100 μl of MetOH and 100 μl of buffer H_2PO_4 and 50 μl were injected into the column. The recovery of quercetin with this protocol was more than 80%.

2.2.3. HPLC equipment

The HPLC equipment utilized for analysis was a BAS CC-5E