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Polyphenol-rich beverages promote a sustainable and renewable generation of energy and prevent neurotoxicity

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Polyphenol-rich beverages, formulations 1 and formulation 2 composed of herbal and berry extracts were developed. Their impact on adenosine triphosphate (ATP) responses was investigated *in vitro* and compared to that of well-known commercial drinks (Red Bull, Coca-Cola, Antioxia, Tetley green tea). Results show that mitochondrial activity, intracellular and extracellular ATP responses are impacted upon the exposure to polyphenol-rich beverages to promote a sustainable and renewable energy supply in human oral CAL27 mucosal cells. While herbal extracts are important for the renewable energetic activity, berry extracts play a role in regulating energy conservation. Prior exposure of polyphenol-rich beverages to CAL27 cells prevented subsequent neurotoxicity of SH-SY5Y dopaminergic cells undergoing oxidative stress. Formulation 1 was best at modulating ATP and neuroprotective responses. On the other hand, Red Bull caused energy depletion, and did not prevent neurotoxicity. In summary, polyphenol-rich drinks are potential energy drinks that promote a sustainable and renewable generation of energy. In particular, Formulation 1 may be a healthy caffeine-free alternative to energy drinks with side effects, such as energy crashing and neurotoxicity.

Key words: Anthocyanins, adenosine triphosphate (ATP), conservation, dopamine, energy crashing, energy drink, mitochondria, neuroprotection, polyphenols, red bull.

INTRODUCTION

People are increasingly concerned about the products they consume and prefer to choose food products with functional botanical natural ingredients over synthetic ones. For this reason, potential health benefits of various botanical ingredients have been widely studied. The beneficial effects of plants typically result from the combination of secondary products present in plants, such as polyphenols, a structural class of molecules characterized by the presence of large multiples of phenol units. Indeed, polyphenols do not only have proprieties associated with food color and aroma, but they may also play an important role in the prevention of many chronic diseases in humans, such as cancer, diabetes, neurodegenerative and cardiovascular diseases (Sears and Rocordi, 2012; Korkina et al., 2012; Xie et al., 2011; Heim et al., 2002). In addition to provide protection from DNA cleavage, polyphenols can modulate various functions, including hormone regulation, enzyme inhibition, immune regulation, lipid peroxidation, capillary permeability, and membrane strengthening (Acquaviva et al., 2003; Lefevre et al., 2008; Rossi et al., 2003).

Energy drinks are popular among people who are looking for ways to boost their energy level for the optimal accomplishment of their everyday tasks. However, many

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health concerns, partly linked to their high levels of caffeine have been reported, including addiction, cardiovascular diseases, impaired cognition and even death. Thus, considerable efforts are undertaken by the food industry to develop healthy energy drinks that are low in caffeine and that are less damaging to consumers.

Novel natural health products that are polyphenol-rich, Formulation 1 (F1) and Formulation 2 (F2), were developed. They were especially designed to serve as adaptogens. In traditional Chinese medicine, the notion of 'adaptogen' has existed for thousands of years, which refers to the ability to regulate the various body functions and increase energy. It was proposed that polyphenols act as adaptogens (Stevenson, 2012). The role of our adaptogenic formulations in energy modulation was investigated to evaluate their potential as energy drinks.

In all forms of life, adenosine triphosphate (ATP), produced in mitochondria, the powerhouse of the cell and most complex cellular organelle is the useable form of chemical energy for the majority of basic metabolic processes (Boyer, 1998). ATP level is a crucial parameter of energy homeostasis, a balanced state ensuring the health and wellness. Levels of ATP are modulated by the diet, which directly affects mitochondrial activity. Most ATP produced by cells is generated from the oxidative phosphorylation process using simple and complex sugars or lipids as a source of energy. Smaller amounts of ATP are generated from the reaction of glycolysis in the cytosol of the cell. Cells stabilize their energetic potential by adjusting the rate of ATP synthesis to the state of energy demand (Fitz, 2007). Optimal metabolic functions can be achieved by consuming food ingredients having the ability to boost ATP responses.

While intracellular ATP (ATPi) is the main energy source required for most intracellular reactions, extracellular ATP (ATPe) released by cells can act as a signaling molecule and influence numerous biological processes, including platelet aggregation, vascular tone, neurotransmission (peripheral and central), cardiac function, muscle contraction, pain and immune responses, male reproduction, fertilization and embryonic development (Gordon, 1986; Volonte et al., 2003; Burnstock, 2006; Le Feuvre et al., 2002; Ostrom et al., 2000). ATPe has dramatic cytotoxic properties and may be involved in P2X7-mediated neurodegeneration (Le Feuvre et al., 2002). Furthermore, ATPe can excite gustatory primary afferent fibers and adjacent cells in taste buds (Huang et al., 2009) known to be innervated by gustatory afferent fibers projecting into the brain, especially the mesolimbic dopaminergic system (MLDS) involved in reinforcement, reward and motivation, in addition to its other functions in motor, mood, stress and addiction.

Dopamine (DA) is the principal neurotransmitter responsible for MLDS activity. Therefore, ATPe may affect the dopaminergic system and should be explored to prevent addiction. In this study, the ability of various beverages, including F1 and F2, to modulate ATP responses in human oral mucosal cells to promote energy homeostasis was investigated. Results show that polyphenols-rich beverages, especially F1, cause a sustained and renewable energy lift in oral epithelial cells, and prevent neurotoxicity of dopaminergic cells.

MATERIAL AND METHODS

Reagents

F1 and F2 are natural health products sold under JustBio's trademarks that were developed in our laboratory. F1 helps relieve nervousness due to mental stress, and F2 helps maintain cardiovascular functions. Concentrated shots of F1 and F2 can be diluted into water to be consumed as functional beverages. Table 1 describes the ingredients of the beverages used in this study. Herbal extracts were generated from dried herbs purchased from La Clef des Champs (Val-David, Quebec). Berry concentrates used in F1 and F2 (blueberry: 65 Brix, cranberry: 50 Brix, and apple: 70 Brix) were purchased from Fruit d'Or (Villeroy, Quebec) and Vergers Paul Jodoin Inc. (Saint-Jean-Baptiste, Québec).

Cell cultures

The human oral cancer cell line CAL27 was purchased from the American Type Culture Collection (ATCC CRL-2095, Massachusetts US). Cells were cultured in Dulbecco's modified eagle medium (DMEM) high-glucose 4.5 g/L culture medium supplemented with 2 mM of L-glutamine, 1 mM of sodium pyruvate, 10% (v/v) fetal bovine serum and 100 µg/ml Penicillin/streptomycin. All cultures reagents were purchased from Thermo scientific Hyclone, Ottawa, Ontario, Canada. CAL27 cells were plated 24 to 48 h prior to the assay in 96 well plates at a density of 20,000 to 30,000 cells per well. SH-SY5Y, human neuroblastoma exhibiting moderate levels of dopamine beta hydroxylase activity (ATCC CRL-2266) were grown in Eagle's minimal essential medium (EMEM)/F12 culture medium containing 10% (v/v) fetal bovine serum. To determine the impact of mucosal treatments on dopaminergic submucosal cells, CAL27 cells were first treated with various beverages for 24 h and their conditioned media was then transferred onto SH-SY5Y cells which were seeded in 96-well plates.

Polyphenols and anthocyanins levels

Polyphenols levels present in the beverages studied were measured by spectrophotometry using an adaptation of a protocol already described by Grubesic et al. (2005). Extraction for the total amount of polyphenols was performed by adding 50% Folin (Sigma-Aldrich) reagent and 20% sodium carbonate solution to diluted samples for 1 h. Absorbance was measured at 760 nm using a Synergy Biotek HT reader. Gallic acid (GA) was used as positive control, and results were expressed as mg equivalent GA per 100 ml of samples. Anthocyanin levels were measured by a differential-pH spectrophotometric method. An aliquot of each sample were mixed with pH 1.0 buffer prepared by dissolving KCl into a 0.2 N HCl solution. At pH 1.0, anthocyanins exist in the

Table 1. Ready–to-drink beverages used and their ingredients.

Beverage	Ingredient
Formulation 1	Lemon balm (*Melissa officinalis* L.), Skullcap (*Scutellaria lateriflora* L.), maple syrup, blueberry concentrate, cranberry concentrate, and natural aroma
Formulation 2	Hawthorn (*Crataegus oxyacantha* L.), Skullcap (*Scutellaria lateriflora* L.), maple syrup, cranberry concentrate, apple concentrate, and natural aroma
Red Bull	Taurine, glucuronolactone, caffeine, niacin (niacinamide), pantothenic acid (calcium d-pantothenate), vitamine B6 (pyridoxine HCl), riboflavine, vitamine B12 (cyanocobalamine), sucrose, glucose, citric acid, inositol, and natural aroma caramel
Coca-Cola original	Glucose-fructose, Coca-Cola mix, caramel color, phosphoric acid, natural flavor, and caffeine
Antioxia	Fruit juice from concentrates (grape, apple, pomegranate, cranberry, blueberry, lemon, elderberry, blackberry), natural flavor, and ascorbic acid (Vitamin C)
Tetley green tea	Green tea (from real brewed green tea concentrate), raw sugar cane, pomegranate juice from concentrate, natural flavor, citric acid, ascorbic acid (vitamin C), and sodium citrate

colored oxonium or flavylium form. Absorbace at 510 nm was measured. Samples were mixed also with pH 4.5 buffer prepared by dissolving sodium acetate in deionized water and by adjusting the pH to 4.5 with HCl. At pH 4.5, anthocyanins are predominantly in the colorless carbinol form. Absorbance at 510 nm was also measured at pH 4.5. The difference in absorbance was proportional to the anthocyanin content. Anthoncyanin analyses were carried out by TransBIOTech (Lévis, Canada).

Mitochondrial activity

CAL27 were seeded at a density of 20,000 to 30,000 cells per well in 96-well tissue culture plates. Colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was dissolved in Hyclone, balanced salt solution, with calcium and magnesium, without phenol red (HBSS) buffer to obtain a final concentration of 0.5 mg/ml per well. Cell plates were incubated for 2 h at 37°C. Conditioned medium was then removed, and acid-isopropanol (0.04 N HCl in isopropanol) was added to all wells. Plates were read at 530 nm/630 nm in a Synergy Biotek HT reader. The amount of dark blue crystals determined by spectrophotometry serves as an estimate for the number of mitochondria and hence the number of living cells in the test sample. This method is an adaptation of a previously described assay (Mosmann, 1983).

ATP responses

CAL27 were seeded at a density of 30,000 cells per well in 96-well tissue culture plates and were incubated in humidified incubator at 37°C for 2 days to allow cell attachment and stable cell growth. After two days of incubation, culture medium was aspirated and the cells were washed and incubated with Hank's buffered salt solution (HBSS) for 20 min. After HBSS incubation, cells were exposed to a first dose of beverages (diluted 1 in 24) for a period of 3 min. After 3 min, successive doses of beverages (diluted 1 in 6) were added for a total of 5 doses and a volume of 100 µl in each well. CAL27 cells were incubated for 25 min. After incubation, the supernatant was aspirated and placed in a second 96-well plate for measurement of ATPe. HBSS was added to each well of the first plate and was frozen at -80°C for cell lysis. Cell lysates were obtained through a freeze-thaw cycle. Levels of ATPi and ATPe were measured using the ATP determination kit from Molecular Probes purchased from Invitrogen (Life Technologies Inc., Burlington, Ontario). Levels were detected by luminescence at 560 nm with a Synergy HT Biotek reader.

ROS production

Cellular levels of reactive oxygen species (ROS) were obtained by measuring the oxidation of 5-(and6)-chlromethyl-20,70-dichlorodihydroflurescein diacetate (CM-H_2DCFDA; Invitrogen), a cell-permeant indicator. SH-SY5Y were seeded in 96-well plates at a cell density of 20,000 cells per well. Cells were treated for 1 h with 5 µM CM-H_2DCFDA dissolved in HBSS. After removing the CM-H_2DCFDA solution, cells were treated with samples prepared with ROS buffer (HBSS containing 2% FBS). After 30 min of exposure with samples, a first reading was taken using the Synergy HT Biotek plate reader. Various concentrations of 2,2'-Azobisisobutyramidinium chloride (AAPH) (40, 16, and 6.4 mM) were added. Plates were read at 485 nm/530 nm every 30 min for 2 h. The fluorescence intensity is an indicator of H_2O_2 intracellular level, so values were expressed in Relative fluorescence unit (RFU).

Salivary DA

Saliva samples were collected from 7 healthy individuals (men and women) who do not smoke, have no addictions, normal weight, exercise at least 2 to 3 times a week. Volunteers were given 20 ml-size samples to drink on separate days. Before swallowing,

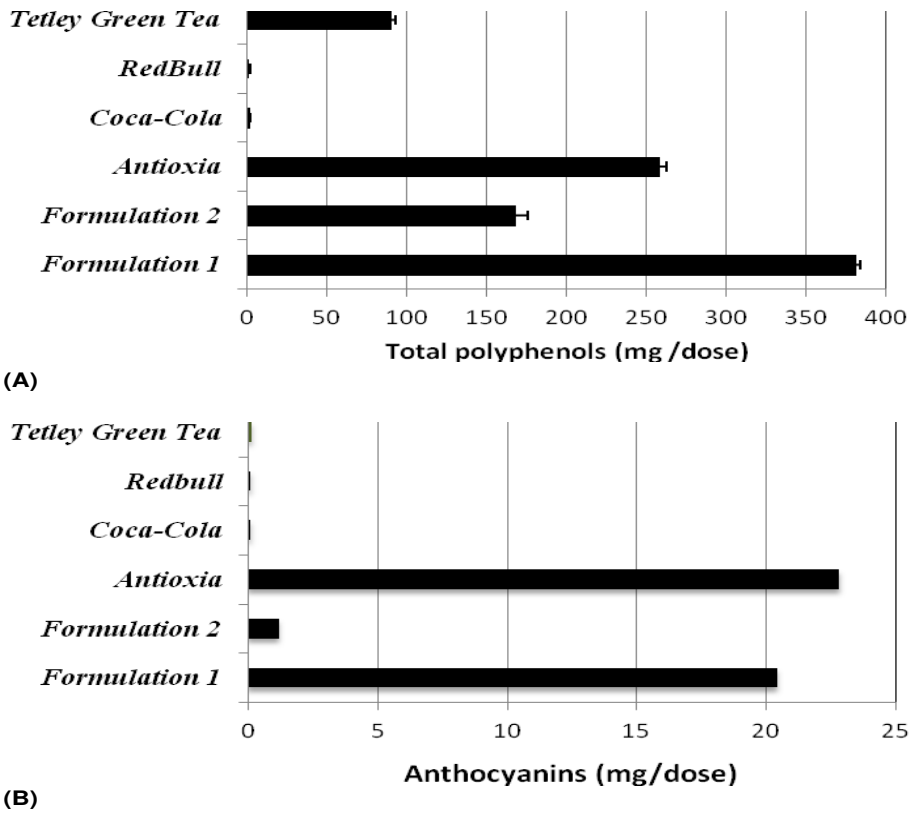


Figure 1. Polyphenol and anthocyanin levels in beverages studied. A. Polyphenol levels in formulations studied. Values are expressed as mean mg/dose +/- SEM of n=6. Gallic acid was used to make the standard curve. B. Anthocyanin levels in formulations studied. Values are expressed as mean mg/dose.

samples were kept in the mouth for at least 15 to 20 s, after which saliva was collected over the next 5 min. Saliva samples were frozen immediately. Levels of human DA from human saliva were measured using an Enzyme-linked immunosorbent assay (ELISA) kit (Genway, CA, USA).

Statistical analyses

Statistical analysis was performed using GRAPH PRISM software. Experiments were done in triplicate. All data are presented as mean ± standard error of mean (SEM). Statistical analyses were done using a Dunnett's multiple comparison one-way analysis of variance (ANOVA) test to compare multiple experimental groups, and using an unpaired *t*-test for results shown in the upper row of Figure 2. Results were considered significant when *$p \leq 0.05$, **$p \leq 0.01$ or ***$p \leq 0.001$.

RESULTS

F1 and F2 are rich in polyphenols

As a result of polyphenols being potential functional molecules, levels of polyphenol of F1 and F2 for one dose were measured and their levels were compared to other beverages. Figure 1A shows that F1 (381 mg/dose) contained the highest levels of polyphenols, followed by Antioxia (258 mg/dose), a wild-berry juice already known to be polyphenol-rich and F2 (180 mg/dose). Tetley green tea (90 mg/dose) had low levels of polyphenols whereas Red Bull and Coca-Cola contained no detectable levels of polyphenols. Anthocyanins levels were detected in F1 and F2. The amounts of anthocyanins per dose present in various beverages are shown in Figure 1B. F1 and Antioxia had the highest levels of anthocyanins with 17 and 19 mg/dose, respectively. F2 had very low but detectable levels of anthocyanins, whereas Red Bull, Coca-Cola, and Tetley green tea did not contain anthocyanins.

Polyphenol-rich beverages regulate ATP responses

The ability to maintain a rate of ATP production in the intracellular environment is a crucial parameter of cellular homeostasis and the cell viability. We tested the capacity of various beverages to generate ATPi in oral human

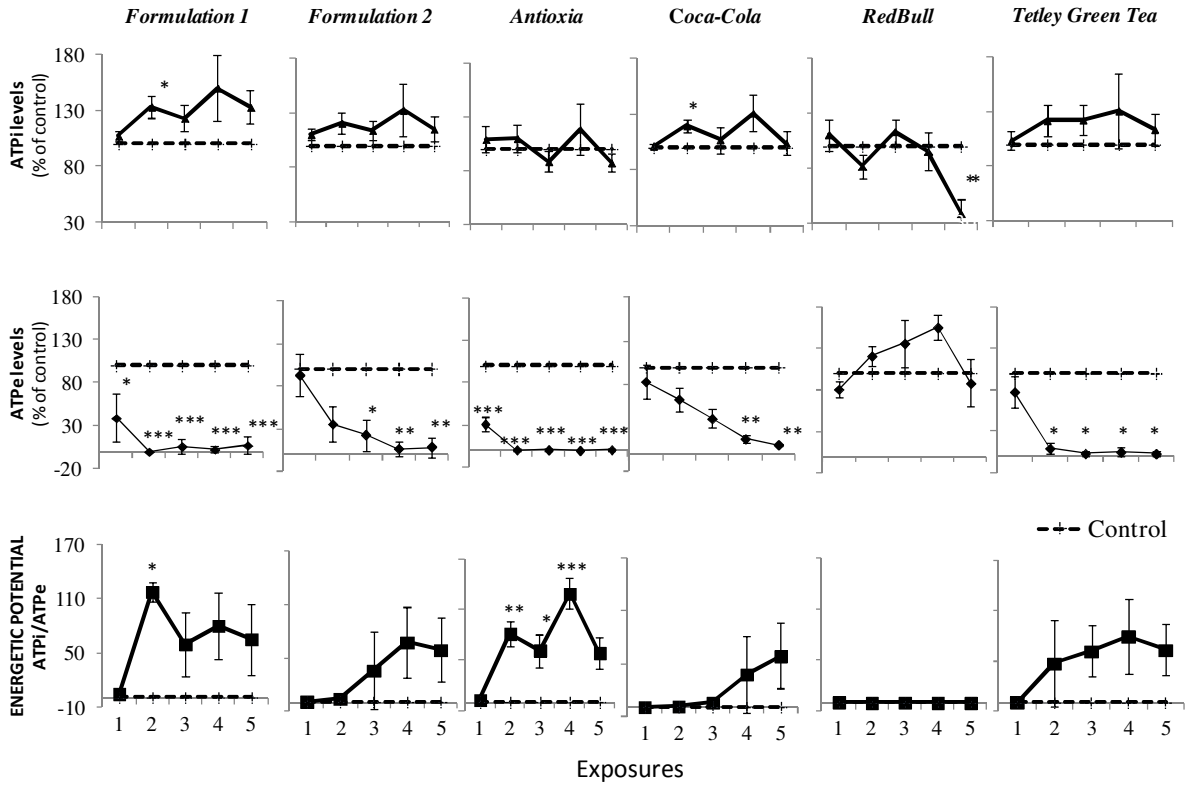


Figure 2. Effects of various beverages on ATP responses in CAL27 oral mucosal cells. Impact of beverages on ATPi (upper row) of CAL27 cells exposed up to 5 times to various beverages is shown. Data from three separate experiments (n = 3) are expressed as percent of HBSS-treated control cells. Values were normalized to control levels (100%), are expressed as mean of normalized ATP levels ± SEM. Paired t-test were performed to determine the level of significance as shown by *p ≤ 0.05. The middle row shows levels of ATPe obtained following exposures to beverages. Values of three separate experiments (n = 3) were normalized to control levels, and are expressed as mean ± SEM of normalized ATP levels produced. In the bottom row, ATPi/ATPe ratios were calculated to show the impact of various beverages on the overall energetic potential of CAL27 cells. Results in middle and bottom row were significantly different from control as shown by *p ≤ 0.05, **p ≤ 0.01 or ***p ≤ 0.001.

CAL27. ATPi levels were normalized to control, and values are expressed as percentage of control. Figure 3 shows in the upper line that ATPi levels of CAL27 were significantly enhanced by F1 after a second exposure and this increase was sustained, suggesting that F1 induces sustainable ATPi production. Coca-Cola significantly enhanced intracellular ATP after two exposures, but this response was not sustained. F2, Antioxia, and Tetley green tea did not significantly modulate ATPi levels. ATPi levels were strongly reduced by five exposures of Red Bull, indicating that it is an inhibitor of ATP production.

ATPe levels were measured from the conditioned culture media collected, following exposure to beverages tested. ATPe levels were normalized to control and values are expressed as percentage of control. Results in Figure 2 show in the middle row that F1 and Antioxia, the beverages containing the highest concentrations of polyphenols and anthocyanins, inhibited ATP secretion of CAL27 upon their first exposure to the beverages. This inhibition was dose-dependent and was maintained after 5 exposures. Blocking ATP secretion may be important in energy conservation. F2, as well as Coca-Cola and Tetley green tea, inhibited ATP secretion from CAL27 cells in a dose-dependent manner, suggesting that these beverages may also play a role in energy conservation. In contrast, Red Bull enhanced ATPe levels in a dose-dependent manner, and the increase was significant after four exposures, indicating that Red Bull induces ATP secretion from CAL27 cells, draining cells from ATPi required for intracellular reactions.

To measure the overall energetic potential, ATPi levels were divided by ATPe levels to obtain ATPi/ATPe ratios. Figure 2 shows in the bottom row that F1 and Antioxia, both polyphenol-rich beverages, are best at promoting the energetic potential of mucosal cells, causing a

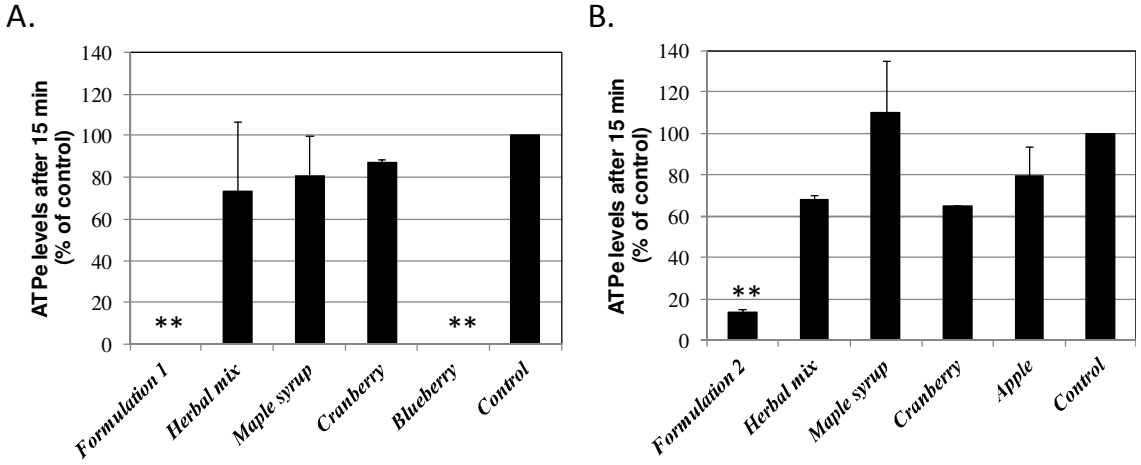


Figure 3. Blueberry of F1 inhibits ATP secretion. (A) The impact of the individual ingredients of F1 on ATP secretion of Cal27 cells was compared to that of F1 itself. (B) Ingredients of F2 were exposed to CAL27 to measure ATP secretion. Ingredients act in synergy to inhibit ATP secretion. ATPe levels were normalized to untreated Values are expressed as mean ± SEM of n = 2. Results were significantly different from control as shown by **p ≤ 0.01.

significant and sustainable energy lift. After 4 exposures, F2 and Tetley green tea were comparable at enhancing the energetic potential of mucosal cells. By inducing ATP secretion and blocking ATP production, Red Bull clearly prevented cells from boosting their energetic potential, suggesting that this beverage cannot be a sustainable solution for long-term energy needs. Finally, Coca-Cola also slightly enhanced the energetic potential of mucosal cells, but only after their fifth exposure. In summary, Figure 2 demonstrates that polyphenol-rich beverages could be sustainable energy boosters.

Blueberries promote energy conservation

The effect of individual ingredients of F1 on ATP secretion was investigated. Figure 3A shows that the blueberry concentrate inhibits ATP secretion as much as the whole beverage (F1) when compared to control (equal to 100%), suggesting that blueberry is responsible for the ATP secretion inhibition of F1. Because F1 contains the highest concentration of anthocyanins, this data suggests that anthocyanins from blueberries may play an important role in energy conservation. Results obtained with ingredients of F2 (Figure 3B) demonstrate that the combination of the different ingredients is required for the ATP secretion inhibition by F2. Despite the presence of low anthocyanin levels in F2, probably due to the cranberry concentrate, the cranberry concentrate was not enough to inhibit ATP secretion. In contrast to blueberry, cranberry is not a potent inhibitor of ATP secretion.

F1 is best at inducing a sustainable energy lift in stressed cells

In CAL27 cells treated with 40 mM of AAPH, Figure 4A shows that F1 and F2 significantly enhanced mitochondrial activity of stressed cells after one exposure only, and this impact was sustained up to three exposures. Antioxia and Coca-Cola also enhanced mitochondrial activity after one exposure, but this was not sustained after subsequent exposures (Figure 4A). Tetley green tea increased mitochondrial activity only after the third application of the beverage, suggesting that it acts more slowly than F1 and F2, or that a higher dose of this drink is required to enhance mitochondrial activity in cells undergoing oxidative stress. In contrast, Red Bull inhibited mitochondria activity of stressed CAL27 cells in a dose-dependent manner. Since MTT value is also measure of cell viability (Mosmann, 1983). this result suggests that Red Bull causes mucosal toxicity by reducing cell viability of stressed CAL27 cells. To determine the effect of oxidative stress on the energetic potential of CAL27, ATPi/ATPe from non-treated cells was subtracted from that of AAPH-treated cells to obtain the net energetic potential of cells. Data shown in Figure 4B indicates that F1 is best at inducing an energy lift in stressed cells after 5 exposure, followed by Tetley green tea and F2. In contrast to what was observed in normal cells (Figure 2B), Antioxia had much lower impact than F1 on the energetic potential of stressed cells (Figure 4B). Red Bull and Coca-Cola had little or no impact on the sustained energetic potential of stressed cells.

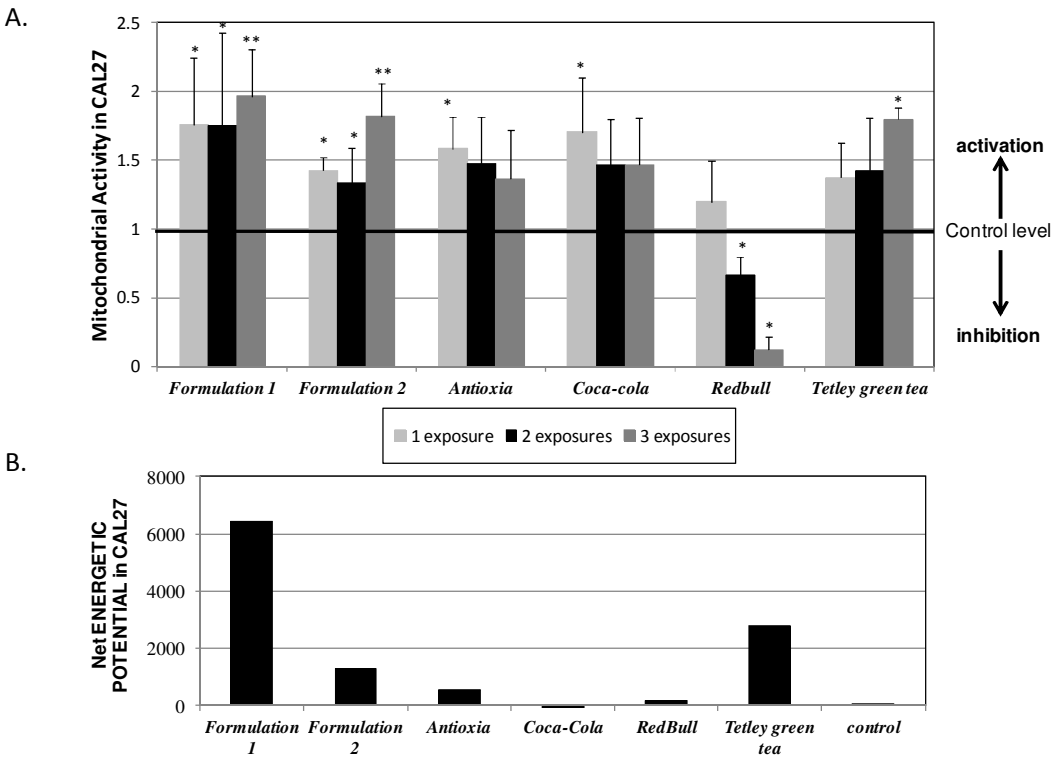


Figure 4. F1 is best at inducing an energy lift in cells undergoing oxidative stress. (A) The impact of various beverages on mitochondria activity of cells treated with 40 mM AAPH was determined. MTT results were normalized to control levels. Values are expressed as mean +/- SEM of n = 3. Results were significantly different from control as shown by *p ≤ 0.05, or **p ≤ 0.01. (B) The net energetic potential was obtained by subtracting the energetic potential of healthy cells (without AAPH treatment) to that of stressed cells (treated with 40 mM AAPH) after 5 exposures. Data was normalized to control, and values are expressed as differences of mean values for n = 3.

Herbal extracts promote a renewable generation of energy

To measure the impact on the renewable generation of energy, mitochondrial activity and ATP responses were measured from the same CAL27 cells receiving two treatments of various beverages on two consecutive days. Results were normalized to control levels post-treatment on the second day. F1, F2, Coca-Cola or Tetley green tea had no effect on mitochondrial activity (Figure 5A). However, Antioxia and Red Bull both inhibited mitochondrial activity in a dose-dependent manner (Figure 5A), suggesting their toxic effect in mucosal cells. Polyphenol levels present in beverages do not correlate with the impact on mitochondrial activity since F1 and Antioxia, containing the highest levels of polyphenols (Figure 1), had differential effects on mitochondrial activity (Figure 5A). This data suggest that herbal extracts present in F1 may be responsible for mucosal protection. The impact for the repeated use of beverages on the energetic potential of CAL27 cells was also determined using ATPi/ATPe ratio. The data clearly shows that Antioxia and Red Bull inhibit the generation of renewable ATP (Figure 5B), whereas F1, F2, Coca-Cola and Tetley green tea enhanced the energetic potential of mucosal cells. Thus, we conclude that the addition of herbal extracts in polyphenol-rich beverages may be required to promote a renewable generation of ATP and to prevent mucosal toxicity.

Polyphenol-rich beverages protect dopaminergic cells

ROS produced from oxidative stress plays an important role in mitochondria-mediated cells death (Orrenius, 2007) Thus, ROS levels can serve as markers of cell toxicity. ROS production of dopaminergic SH-SY5Y neurons treated with 40 mM AAPH was measured after being exposed to conditioned media of CAL27 cells treated with various beverages. Results demonstrate that F1, F2, and Antioxia provided neuroprotection to dopaminergic cells

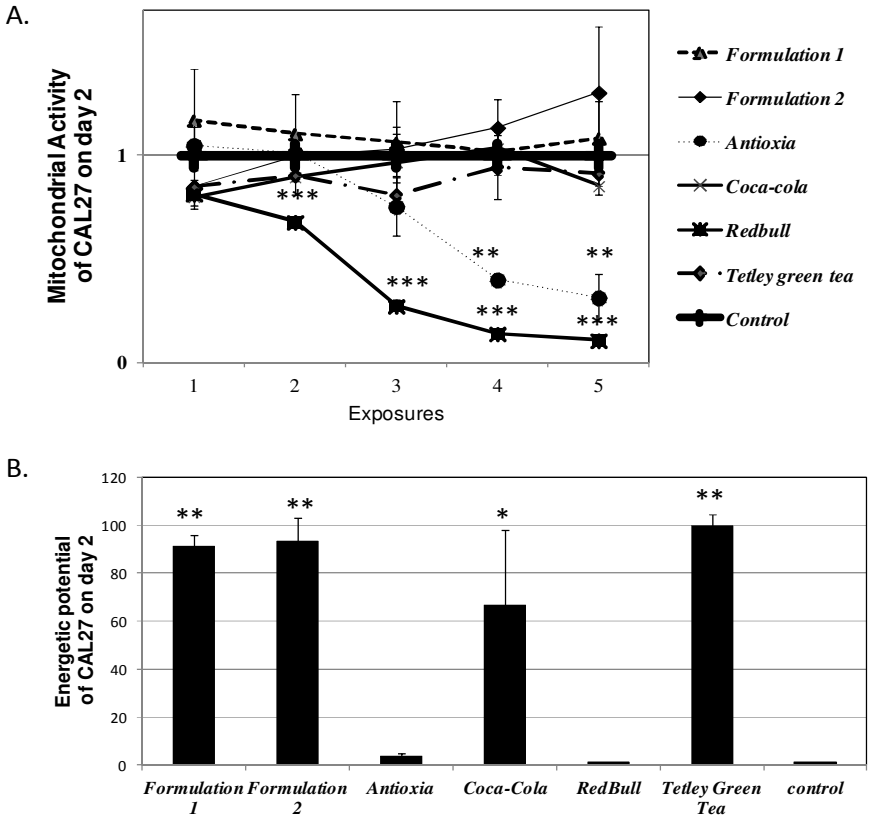
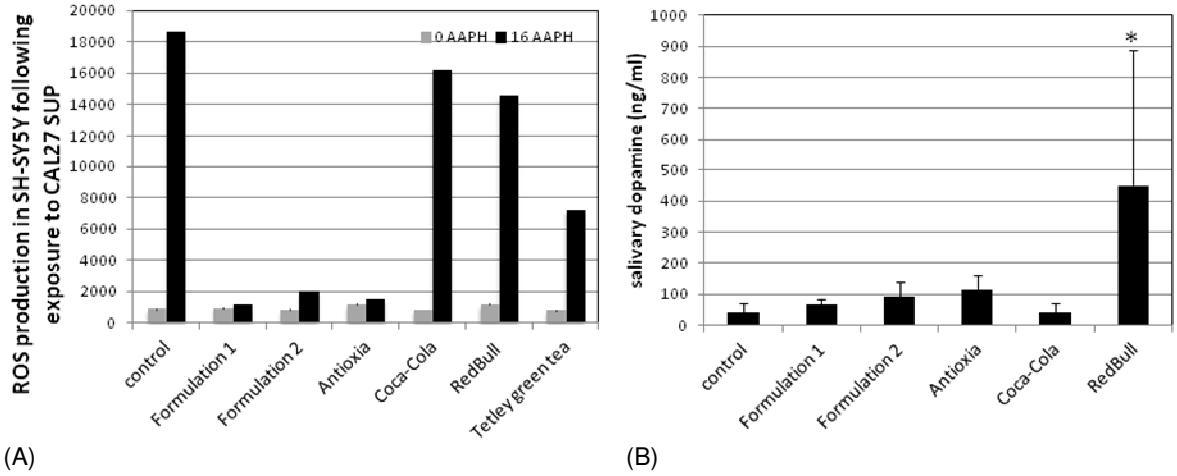


Figure 5. The impact of various beverages on renewing energy. MTT results measuring mitochondrial activity from CAL27 cells treated twice on two consecutive days with various beverages were obtained. MTT results were normalized to control levels. MTT results are directly proportional to cell viability. Values are expressed as mean ± SEM of n = 3. (B) To measure the energetic potential, ATPi/ATPe ratio were obtained on the second day of treatment of CAL27 cells after 5 exposures with various beverages. Values are expressed as mean ± SEM of n = 3. In both A and B, results were significantly different from control as shown by *p ≤ 0.05, **p ≤ 0.01 or ***p ≤ 0.001.

by inhibiting ROS production, while Coca-Cola and Red Bull did not (Figure 6A). Tetley green tea had a low neuroprotective activity compared to the other polyphenol-rich beverages. To measure the impact of beverages on the human submucosal dopaminergic system, levels of DA were detected in saliva of human subjects five minutes after drinking beverages. Figure 6B show that Red Bull significantly enhanced levels of salivary DA compared to control (water), whereas all other beverages tested did not. This data suggests that, in contrast to Red Bull, polyphenol-rich beverages do not induce an addictive response.

DISCUSSION

We provide evidence for the first time that polyphenols regulate ATP responses by inducing, through the oral intake of polyphenol-rich beverages, a sustainable energy lift in mucosal cells. Results show that polyphenol-rich can enhance ATP production and block ATP secretion simultaneously. The impact dietary intake of polyphenols on energy regulation has been proposed through a link with the hypothalamic neuropeptide systems (Panickar, 2013). Here, we propose that polyphenols can directly regulate energy regulation by modulating ATP responses. It is possible that the stimulation of ATP production results in the acceleration of ATP synthesis by increasing mitochondrial electron transport and/or decrease ATP consumption (or degradation). The optimization of body functions such as muscle contraction and recovery, mental focus, and maintenance of the immune system require a constant energy supply. Baicalein and baicalin are two major



(A) (B)

Figure 6. Polyphenol-rich beverages protect the dopaminergic system. (A) Human dopaminergic cells were treated with conditioned media from CAL27 cells exposed to various beverages for 24 h. ROS production of SH-SY5Y was induced with 16mM AAPH (n = 1) or without AAPH (n=3) to determine the impact when cells are undergoing oxidative stress. Data show that mucosal exposure of polyphenol-rich beverages (F1, F2, Antioxia) inhibited ROS production in dopaminergic cells that may be present submucosally. (B) Levels of salivary DA detected 5 min after drinking 20 ml of various beverages in healthy human individuals are shown. Values are expressed as mean ± SEM of salivary DA ng/ml of at least n = 6. Results were significantly different from control as shown by *$p \leq 0.05$.

flavonoids of skullcap (*Scutellaria baicalensis* Georgi or *Scutellaria lateriflora)*. A study suggests that baicalin or baicalein increases cellular ATP levels of HIT-T15 cells (Li et al., 2012). Thus, skullcap extracts contained in F1 is likely to regulate ATP responses.

To understand the energy potential of F1, it would be also interesting to study ATPi production using other cell types. Wong et al. (2011) investigated the energizing properties of plants used in modern Chinese medicine, using H9c2 cardiomyocytes, and they demonstrated properties of 'Yang-invigorating' herbs to stimulate their mitochondrial activity. Red Bull decreases ATPi after 5 doses (Figure 2), causing cellular energy depletion. Certain factors can cause ATP depletion, including severe oxidative stress (Ahmad et al., 2004). There is also increasing evidence for the physical alteration of the biological membrane as a major factor in the evolution of irreversible injury during ATPi depletion (Florine-Casteel et al., 1991). It has been shown that there is a sequence of specific intracellular events linked to cell death by necrosis (Figure 1) (Golstein and Kroemer, 2006). This sequence includes the first signs of mitochondrial dysfunction such as ROS production by mitochondria and mitochondrial swelling, the decrease in intracellular ATP, loss of Ca^2 + homeostasis, perinuclear clustering of organelles, activation of some proteases lysozymes rupture, and finally rupture of the plasma membrane.

A role for polyphenols in energy conservation is proposed because polyphenol-rich beverages have the ability to block ATP secretion. Mechanisms of energy conservation are not well understood, but it is known that energy conservation can be triggered by various meditation techniques. For example, it is known that highly experienced yogi have the capacity to meditate for very long periods of time without eating, possibility by enhancing their energy conservation capacity. Meditation is known to affect the autonomic nervous system, causing heart rate variability (Servant et al., 2009) and to activate the sympathetic nervous system, and subsequent catecholamine/cortisol release for controlled stress responses. The innate immune system can also be impacted by meditation, and this modulates inflammatory responses (Kox et al., 2012). With its ability to restore and maintain a general state of homeostasis, meditation can influence the fate of diseases such as the metabolic syndrome and coronary heart disease (Paul-Labrador et al., 2006).

Red Bull induces ATP secretion (Figure 2) and enhances mucosal cell death (Figure 3), suggesting a role in mucosal toxicity. It has long been known that ATPe may be a mediator of cytotoxic cell-dependent lysis (Francesco et al., 1990). Since massive extracellular release of ATP often occurs after metabolic stress, brain ischemia and trauma, ATPe may be involved in the etiopathology of many neurodegenerative conditions. Studies on immune cells have demonstrated that ATPe can act as a potent stimulus for the maturation and release of interleukin-1β via activation of purinergic P2X7

receptors (Le Feuvre et al., 2002). ATPe is also known to be toxic to primary neuronal dissociated cells and organotypic neuronal cultures from cortex, striatum and cerebellum. Therefore, it is likely that excess amount of ATPe can be damaging to surrounding neuronal cells.

Protective effects of mitochondrial activity by polyphenol-rich beverages can be explained by their antioxidant properties or their capacity to boost ATP generation. First, mitochondria are the major sites of cellular ROS production and also targets of ROS. Mitochondrial DNA, proteins, and lipids in the inner membrane of mitochondria are thus vulnerable to oxidative damage by ROS. Maintaining a balance between ROS levels and antioxidant molecules concentrations prevent development of generalized mitochondrial dysfunction and poor energy metabolism (Halliwell, 1996). Antioxidants protect cell membrane integrity, contributing to the resistance against assaults by ROS or by autolytic enzymes during the process of irreversible cell injury (Wu et al., 1996). Interestingly, our laboratory has demonstrated that F1 and F2 have strong antioxidant proprieties by decreasing ROS concentration produced by CAL27 cells (unpublished observation). These antioxidant properties could be explained by the presence of polyphenols in *Melilotus officinalis*, which was shown to have the highest antioxidant activities when compared to other plant extracts (Picada Pereira et al., 2009). Further investigation is necessary to determinate the mechanisms underlying the link between this antioxidant capacity and the modulation of ATPi production.

Results show that the blueberry concentrate alone inhibits ATP secretion to the same extent as F1 (Figure 3A). The effect of blueberry extract on ATP secretion has been demonstrated in a neurodegenerative model induced by amyloid-β peptide where acute ATP leakage was prevented (Fuentealba et al., 2011). Blueberries (*Vaccinium* spp), blackberries (*Rubus* L. hybrids), and black currants (*Ribes nigrum* L.) are rich sources of dietary anthocyanins. High levels of anthocyanins in berries are thought to play an important role in human health and disease prevention due to their powerful antioxidant activity (Zafra-Stone et al., 2007).

Indeed, anthocyanins are increasingly studied for their physiological roles in protecting higher plants against destructive oxidative damage (Wang et al., 2009; Prior et al., 2003). The amounts and distribution of anthocyanins in berries differ, depending on their plant species, cultivation conditions, and producing districts.

Consequently, the antioxidant activity may be different among various berry extracts (Connor et al., 2002). For example, the degree of ripeness differently affects the concentrations and proportions of the various polyphenols: generally phenolic acid concentrations decrease during ripening, whereas anthocyanin concentrations increase (D'Archivio et al., 2007). Moreover, several studies have shown that the content and antioxidant activities of total anthocyanins and total phenolics in various fruits are highly correlated (Moyer et al., 2002; Wang and Lin, 2000). However, studies using extracts from different fruits and vegetables have suggested that there may be synergic or additive biological effects due to unique combinations of anthocyanins and other phenolics (Bagchi et al., 2004). These differences can depend on the type of cultivar used, since many cultivars and native species of these berries exist, some with substantially higher antioxidant levels than others.

The neurotransmitter dopamine plays a major role in reward processing, regulating reinforcement and motivational behavior, in addition to other functions in motor, mood, stress and addictive behavior. We show that polyphenol-rich beverages, including F1 and F2, provided mucosal protection and subsequent neuroprotection by preventing dopaminergic cells from undergoing oxidative stress (Figure 6A). F1 and F2 are composed of skullcap, known to contain baicalein, which can protect PC12 cells of 6-hydroxydopamine (6-OHDA)-induced damage (Zhang et al., 2012). Thus, it is possible that baicalein plays a role in the neuroprotection of F1 and F2. In contrast to the neuroprotective effect of F1 and F2, Red Bull caused mucosal toxicity and did not protect dopaminergic cells from oxidative stress (Figure 6A). Neurotoxicity of SH-SY5Y by an impairment of mitochondrial ATP synthesis was previously shown using methadone (Perez-Alvarez et al., 2010), suggesting that Red Bull may act through the same pathway to cause neurotoxicity.

Interestingly, DA levels in human saliva were enhanced only after drinking a sip of Red Bull, but not after drinking polyphenol-rich beverages (Figure 6B), suggesting that Red Bull causes the activation of the dopaminergic system that plays a role in addiction. We conclude that polyphenol-rich beverages may protect the dopaminergic system, while Red Bull does not. Further studies in a larger group of individuals will be required to prove this point, and to better understand the impact of polyphenol-rich beverages on the dopaminergic system.

Conclusion

Mucosal ATP responses can be regulated through the intake of polyphenol-rich beverages to ensure a constant supply of energy. Taken together, results summarized in Table 2 shows that F1 is best, compared to other beverages tested, at promoting a sustainable and renewable generation of energy, and at providing mucosal protection and neuroprotection of dopaminergic cells. On the other hand, the well-known energy drink Red Bull did not promote a sustainable and renewable production of ATP

Table 2. Summary of results.

Parameter	F1	F2	Antioxia	Coca-Cola	Red Bull	Tetley green tea
Polyphenol-rich	++++	++	+++	-	-	++
Anthocyanin-rich	+++	+	++++	-	-	-
Sustainable energy lift in healthy mucosal cells	+++	-	++++	-	-	-
Sustainable energy lift in damaged mucosal cells	++++	++	+	-	-	++
Renewable energy lift in healthy mucosal cells	++	++	-	+	-	++
Energy conservation	+++	++	++++	++	-	++
Mucosal protection	+++	++	-	+	-	++
Neuroprotection of dopaminergic cells	+++	+++	+++	-	-	++
Total score	25	14	19	4	0	12

and instead caused an energy crash. We propose that oral intake of polyphenol-rich beverages, especially F1, can act as energy drinks to promote energy homeostasis. Therefore, one should consider polyphenol-rich beverages as alternatives to commercial caffeine-rich energy drinks, such as Red Bull, with toxic effects.

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Abbreviations: ATPe, Extracellular ATP; **ATPi,** intracellular ATP; **DA,** dopamine; **F1,** formulation 1; **F2,** formulation 2; **MLDS,** mesolimbic dopaminergic system; **ROS,** reactive oxygen species; **SEM,** standard error of the mean, **ATP,** adenosine triphosphate.

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