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Full Length Research Paper

Nutrigenomic studies of orange juice and vitamin c on gene expressions in neuronal tissues of Sprague Dawley rats

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Nutrigenomics, a nascent field in nutrition and molecular biology plays a pivotal role in understanding the influence of bioactive dietary components on the structure, integrity and functions of the genome. In this study, the comparative effects of vitamin C (Vit C) and Nigerian local orange juice on the gene expression levels of superoxide dismutase (*sod*), tumour necrosis factor receptor (*tnfr*), *p53* protein (*p53*), estrogen receptor (*esr*) and progesterone receptor (*pgr*) genes were evaluated in rat neuronal tissues. 5 ml/Kg bodyweight of freshly extracted orange juice and 20 mg/Kg bodyweight Vit C were administered orally to Sprague Dawley rats distributed into orange, vitamin C and control groups, respectively. Expressions of *sod*, *tnfr*, *p53*, *esr* and *pgr* genes were measured by relative quantification PCR using SYBR green chemistry. Data were analyzed using Applied Biosystems SDS software 1.4 and Graphpad prism 5.0. A 57.01 ± 6.37 fold significant (*P<0.01*) increase in *tnfr* gene was observed in the orange juice administered male group compared with the vitamin C (2.53 ±0.74 fold change) and control groups. Other genes investigated (*sod*, *p53*, *pgr* and *esr*) also showed different expression patterns but the mean differences between the test groups was not significant (*P>0.05*). Orange juice has been shown to posses the ability to increase significantly mRNA expression levels of *tnfr* gene in the brain neuronal tissues of male rats. The increase is probably mediated by other bioactive components in the orange juice rather than vitamin C.

Key words: Vitamin C, orange juice, relative quantification PCR, gene expression, brain tissue.

INTRODUCTION

Nutrigenomics is the study of nutrition or dietary components on the transcriptome of cells and tissues. This is further described as the influence of genetic variation on nutrition by correlating gene expression or single nucleotide polymorphism with nutrient absorption, metabolism, elimination, and biological effects (Kaput et al., 2007). Several researches in nutrigenomics have shown that apple juice, promegranate juice and red grape juice contain bioactives that can affect gene expression (Soyalan et al., 2011; De-Nigris et al., 2005; Davalos et al., 2009).

However, one of the most susceptible organs to free radical damage is the brain because it contains high amount of lipids (Siegel, 2006). Due to the brain's critical dependence on aerobic metabolism, mitochondrial respiratory activity is higher than in many other tissues thus; increasing the risk of free radical leakage from the mitochondria in which radical damage to the mitochondria in the brain ensues. Oxidative stress increases with age and therefore it can be considered an important causative factor in several neurodegenerative diseases, typical for older individuals (Valko et al., 2007) of which Alzheimer,

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Table 1. Grouping of animals.

Group	Number of animals	Treatment
Group 1	3	Male rats administered distilled water (Control)
Group 2	3	Male Rats administered 5 ml/Kg body weight orange juice
Group 3	3	Male rats administered 5 ml/kg body weight vitamin C solution
Group 4	3	Female Rats administered distilled water (Control)
Group 5	3	Female rats administered 5 ml/kg bodyweight orange juice
Group 6	3	Female rats administered 5 ml/kg bodyweight vitamin C solution.

Parkinson and Huntingtons disease are major examples associated with oxidative stress. There is considerable evidence that many of the neurons that die from neurodegenerative diseases undergo apoptosis (Figiel, 2008).

Oxidative stress may trigger apoptosis by activating membrane associated apoptotic signaling cascades (Cutler et al., 2004). This is achieved by the actions of TNF-α mediated through two distinct cell surface receptors: *TNFR*1 (p55) and *TNFR*2 (p75). Therefore, TNF-α binding to *TNFR*1 may result in either activation of apoptosis or transcriptional activity (MacEwan, 2002; Wajant et al., 2003). *p53*, a tumour suppressor/pro-apoptotic gene becomes activated in response to a myriad of stress types resulting to DNA damage (induced by either UV, IR, or chemical agents such as hydrogen peroxide), oxidative stress, osmotic shock, ribonucleotide depletion, and deregulated oncogene expression (Han et al., 2008). In contrast, amyloid β accumulation in relation to apoptosis in the brain has been linked to increased levels of *p53* and *bax* genes (Mattson, 2000). The importance of superoxide dismutase in the brain cannot be downplayed, manganese-containing superoxide dismutase (Mn-SOD or SOD2) - a specific antioxidant enzyme for superoxide, is a primary cellular defense enzyme involved in protecting cells from oxidative stress (Chan, 1996). Previous reports by Murakami et al. (1998), Fujimura et al. (1999) and Kim et al. (2002) show that Mn-*sod* deficient mice (*sod*2-/-) had increased infarct volume and apoptosis after cerebral ischemia.

Over-expression of Mn-*sod* provided neuro-protection alongside cerebral ischemic reperfusion (Maier et al., 2006). Progesterone, as estrogen, affects many functions of the central nervous system (Chen et al., 2008; Stein et al., 2008). It plays an important role in promoting and enhancing repair after traumatic brain injury and stroke, and there has been growing evidence that progesterone treatment may be safe and effective for traumatic brain injury and other neural disorders in humans (Wang et al., 2011).

Citrus sineensis (oranges) are good sources of vitamins, especially vitamin C (Morton, 1987). Ghanim et al. (2010) and Fatemeh et al. (2009) reports that orange also contain bioactives that can affect gene expression. In addition to the antioxidant effect of vitamin C, other components (hesperedine) in orange juice have been shown to prevent oxidative stress and alter the expression of genes. Shin et al. (2004) published a series of genes responding to ascorbic acids treatment of embryonic stem cells. Most of the overexpressed genes belong to the families involved in neurogenesis, maturation, and neurotransmission diseases (Belin et al., 2009). The aim of this study was to comparatively evaluate the neuroprotective effect of the Nigerian orange juice and vitamin C on the differential gene expressions of superoxide dismutase (*sod*), tumour necrosis factor receptor (*tnfr*), *p53* protein (*p53*), and progesterone receptor (*pgr*) genes using brain tissues from Sprague Dawley rats.

MATERIALS AND METHODS

Experimental animals

Twenty (20) Sprague-Dawley rats (156.68 ± 4.72 g), comprising 10 males and 10 females were collected from the animal facility of the College of Medicine, University of Lagos, Nigeria, and handled based on the guidelines/policies of the animal facility ethics. Animals were fed rat chow and water *ad-libitum*. Based on treatments and dosage regimen, animals were divided into six groups of three animals each as summarized in the Table 1. Administration was done three times daily for seven days.

RNA extraction/cDNA synthesis

After the seventh day administration, animals were sacrificed and their brain tissues were collected and stored immediately under liquid nitrogen. Total RNA was extracted using Qiagen RNeasy® extraction kit. Extracted RNA samples were quantified using Nanodrop spectrophotometer. 100ng of normalized RNA samples were converted into cDNA in a reaction volume of 25 ul comprising of 500ng oligodT, 1X Script buffer, 0.1 mM DTT, 1U/µl Rnase inhibitor, 0.4 mM dNTP, 4.0 U/µL reverse transcriptase enzymes. PCR condition was done as described in the Jena Bioscience SCRIPT® Reverse transcriptase kit.

Primer design

cDNA sequences of the study genes- *sod*, *tnfr*, *p53*, pr, and esr were extracted from the NCBI gene bank. Primers were designed to span exon-exon region using Primer 3 tool Software. The primer sequences are shown in Table 2.

Table 2. Generated Primers For each mRNA.

Gene	Accession Number	Primer	Sequence (5'-3')
Estrogen receptor	NM_012689.1	Esrfwd	5'-GCT ATG GAA TCT GCC AAG GA-3'
Estrogen receptor		Esr rvs	5'-GGC AGC TCT TCC TCC TGT TT-3'
Progesterone receptor	NM_022847.1	Prfwd	5'-AAG GAA GAT TCC CGC TTC TC-3'
Progesterone receptor		Pr rvs	5'-GCC CTC GTA ACT TTC GTC TT-3'
Tumour necrosis factor receptor	NM_013091.1	*Tnfr*fwd	5'-GTG CCT ACC CCA GAT TGA GA-3'
Tumour necrosis factor receptor		*Tnfr*rvs	5'-CTT CAA GCT CCC CCT CTT TT-3'
p53 protein gene	NM_030989.3	*P53* fwd	5'-GCG CAC AGA GGA AGA GAA TC-3'
p53 protein gene		*P53* rvs	5'-CAA GGC CTC ATT CAG CTC TC-3'
Beta actin	NM_031144.2	Actbfwd	5'-GGC ATG GGT CAG AAG GAT TC-3'
Beta actin		Act b rvs	5'- ACA TGA TCT GGG TCA TCT TCT C-3'

Relative gene quantitation

Polymerase chain reaction **(**PCR) was performed in a 25 µL reaction volume containing 5 µg of cDNA, 0.2 mM dNTP mix, 1X Complete Buffer (Jena Biosciences), 0.04U/µl High Yield taq polymerase (Jena Bioscience), 0.5 X Sybr green I (Invitrogen, Germany) and 0.5 µM of each target primer pair. Beta actin gene was used as the internal control (endogenous gene). Thermal cycling was done using the Applied Biosystems Real Time PCR 7300 at 94°C for 2 min; 94°C for 30 s; 56°C for 30 s; 72°C for 30 s; and 72°C for 2 min. Step 2 to 4 was repeated 35 times and data were acquired using the Applied Biosystem SDS Software. Dissociation curve analysis was done in order to check for unspecific amplifications.

Data analysis

RT-PCR was done in replicates of atleast three wells for each target gene. Comparative C_t ($\Delta\Delta C_t$) was calculated by subtracting $\Delta C_{t,\ calibrator}$ from $\Delta C_{t\ (treated\ samples)}$. Relative fold changes were determined using the formula $2^{-\Delta\Delta CT}$ as described by Livak and Schmittgen (2001). Data were analyzed using Graphpad Prism 5.0 statistical package. Independent Student *t*-test was used to determine the significance ($P<0.05$) between the mean differences of the test groups (vitamin C and orange juice group).

RESULTS AND DISCUSSION

Orange, a citrus fruit and one of the most abundant and affordable fruit consumed by every population across the whole world has been shown to prevent oxidative stress and modulate the expression of genes that may trigger apoptosis (Fatemeh et al., 2009). In this study, the administration of orange juice caused a non-significant (*P>0.05*) increase in the mRNA expression level of *p53* (males and females) as compared with vitamin C group (Figures 2a and 2b). In the male rats, a 57.01 ± 6.37 fold significant (*P<0.05*) increase was observed (Figure 1a) in the expression levels of *tnfr* gene of the orange administered group. These increases in the expressions of *p53* and *tnfr* may promote apoptosis. Manipulation of the apoptotic functions of these genes constitutes an attractive target for cancer therapy (Kane and Citron, 2009; Martin, 2010). In contrast, over expression of apoptotic genes results in neuronal cell deaths in neurodegenerative disorders, and posses deleterious effects on the body (Shacka and Roth, 2005; Morrison and Kinoshita, 2000).

Studies have revealed that one of the mechanisms by which fruits and vegetables prevent oxidative stress is the alteration in the level of expression of the genes that are involved in the generation and removal of the reactive oxygen species. For example, apple juice and its component polyphenols have been shown to modulate the expression of antioxidant response element (Solayan et al., 2011). Promegranate juice reduced the activation of redox sensitive genes (ELK-1 and p-JUN) and increased extracellular nitric oxide synthase (eNOS) expression (De-Nigris et al., 2005). Superoxide dismutase (sod) is a member of the antioxidant enzymes, superoxide dismutase family that catalyses the spontaneous dismutation of superoxide anion to hydrogen peroxide and oxygen. The orange administered groups (both male and female rats) show non-significant (P>0.05) increase in sod expression levels (Figures 1c and 1d). Solayan et al. (2011) observed that apple juice modulates antioxidant response element genes but the sod1 and sod2 genes were not affected or down-regulated.

Estrogens exhibit their anti-inflammatory and neuroprotective effects in the brain via estrogen receptor α and β (Sarvari et al., 2011). In this study, esr gene showed a non significant (P>0.05) progressive increase in fold change from rats administered vitamin C to rats administered orange juice (Figure 2d). The ability of vitamin C to alter gene expression has been demonstrated by various research studies. Shin et al. (2004) published a series of genes responding to ascorbic acids treatment of embryonic stem cells. Most of the overexpressed genes belong to gene families in-volved in neurogenesis, maturation and neurotrans-mission. Progesterone receptors (PR) are highly ex-pressed throughout the brain and can be found in every neuronal cell types. It therefore has multiple non-reproductive

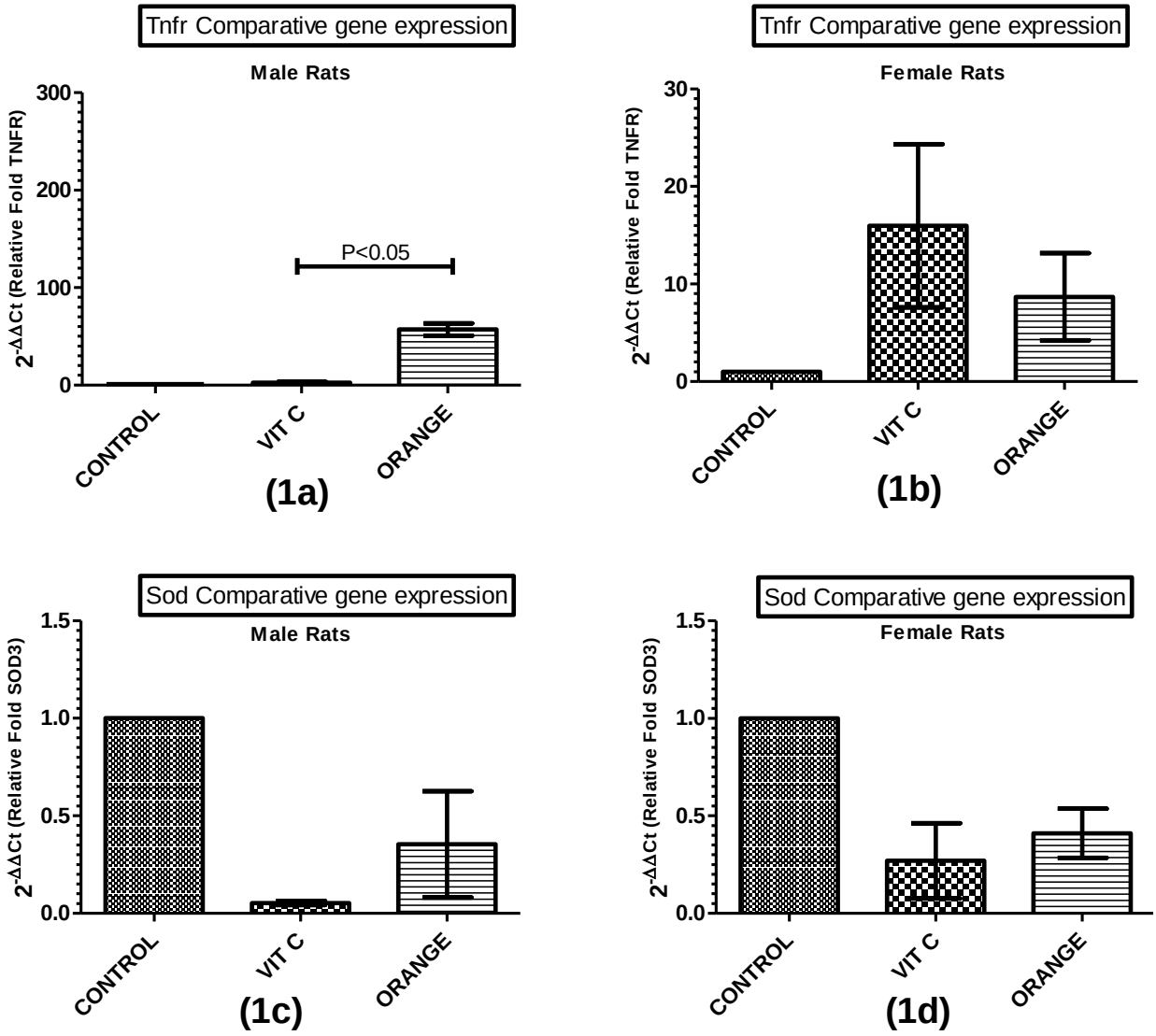

Figure 1. Error bar charts of: (a) the relative *tnfr* gene expressions in brain tissues of male rats; (b) female rats; (c) sod gene expressions in brain tissues of male rats and (d) female rats. Bars represent mean values ± standard error. No. of samples=3. P<0.05 is considered significant.

functions in the central nervous system to regulate cognition, mood, inflammation, mitochondrial function, neurogenesis and regeneration (Brinton et al., 2008). Progesterone receptor mRNA relative expression in this study showed a non significant (*P>0.05*) fold increase in the orange juice group (58.34 ± 21.32) compared with the vitamin C group ($5.06 \times 10^{-4} \pm 2.69 \times 10^{-4}$).

Conclusion

Orange juice has been shown to posses the ability to increase significantly mRNA expression levels of *tnfr* gene in the brain neuronal tissues of male rats. The increase is probably mediated by other bioactive components in the orange juice rather than vitamin C. These findings may have implications in the prevention of cancer.

RECOMMENDATIONS

The bioactive components in orange juice responsible for the modulation of the study genes should be identified and may be used in the prevention of cancer. Further work is necessary to determine the effect of orange juice (or bioactive components) on other genes that are associated with apoptosis, other antioxidant enzymes, and genes associated with the generation of reactive oxygen species.

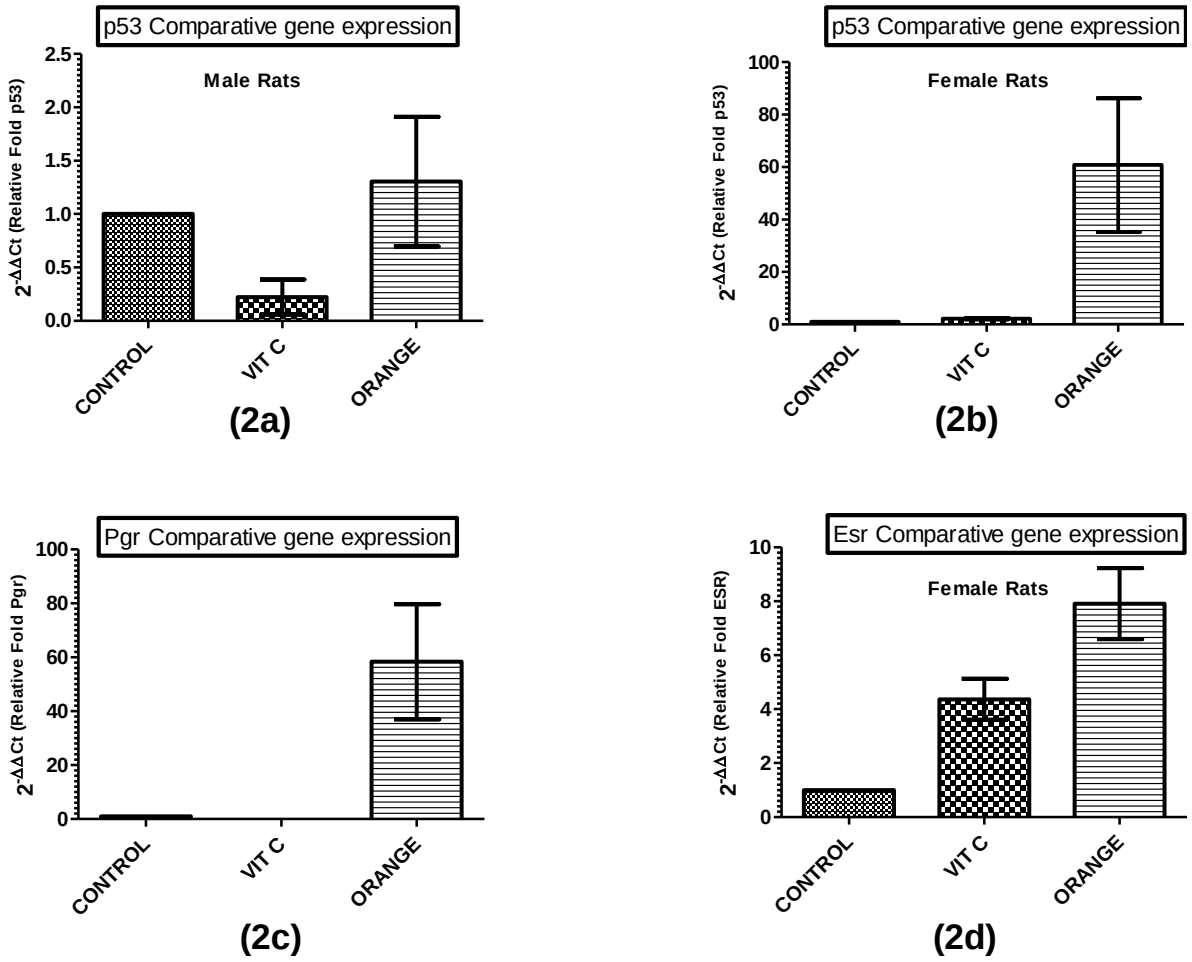

Figure 2. Error bar charts showing the relative *p53* gene expressions in brain tissues of male and female rats in (A) and (B), respectively. (C) Relative *pgr* gene expressions in brain tissues of rats. (D) esr gene expressions in brain tissues of female rats. Bars represent mean values ± standard error. No. of samples=3. $P<0.05$ is considered significant.

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