COMPARATIVE INVESTIGATION OF ORANGE JUICE AND VITAMIN C ON GENE EXPRESSION IN NEURONAL TISSUES OF SPRAGUE DAWLEY RATS

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Abstract:

The comparative effects of Vitamin C and Citrus sinensis (orange) juice on the gene expression levels of superoxide dismutase (Sod), tumour necrosis factor receptor (Tnfr), p53 protein, and progesterone receptor (Pgr) genes were evaluated in rat neuronal tissues. Experimental rats were given 5ml/Kg body weight of freshly extracted orange juice or 20mg Vitamin C /Kg body weight. Control animals were fed with rat chow and water. After 7th day administration, brain tissues were collected in liquid nitrogen and total RNA was extracted using Qiagen Rneasy extraction kit. 100ng of normalized RNA samples were converted to cDNA with Oligo-dT primers. PCR Primer pairs were designed using Primer 3 and NCBI primer blast tool. SYBR green Real time PCR relative quantification was done using High yield Taq Polymerase Kit and SYBR green dye. The orange juice extract contained 15.84mg Vitamin C, 13.67g total sugar, 0.02 mg sodium, 0.15 mg iron, 11.48 mg magnesium, 44.20 mg calcium, 4.76 mg potassium, 0.75 mg zinc, and 22.9 mg phosphorus. Relative quantification gene expression assays in the rats showed a 400 fold change in Tnfr and 38 fold change in p53 gene in the orange administered group in comparison to the Vitamin C administered group and control group. Vitamin C caused a 420 fold change in the expression of Pgr gene in comparison with the orange administered group. Our findings showed that, orange juice administered rats showed higher levels of gene expressions of Tnfr and p53 genes, thereby suggesting brain tumour preventing ability of oranges.

Key words: *Nutrigenomics, orange juice, vitamin C, gene expression, neuronal tissues*

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).

Citrus sinensis (oranges) are good sources of vitamins, especially vitamin C (Morton,1987). Grahim *et al.*, (2010) and Fatemah *et al.*, (2009) reported that orange also contains 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 acid treatment of embryonic stem cells. Most of the overexpressed genes belonged to the families involved in neurogenesis, maturation, and neurotransmission (Belin *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 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). Alzheimer's, Parkinson's, and Huntington's disease are examples of neurodegenerative diseases associated with oxidative stress. Oxidative stress may trigger apoptosis by activating membrane associated apoptotic signaling cascades (Cutler *et al.*, 2004). There is considerable evidence that many neuronal deaths in neurodegenerative diseases are due to apoptosis; examinations of the brains of Alzheimer's, Parkinson's and Huntington's disease patients have revealed evidence for the activation of caspases and regulation of apoptotic proteins, such as Bax, p53, and Par-4 (Matton, 2000).

The aim of this study is to evaluate the comparative neuroprotective effect of orange juice and vitamin C on the differential gene expressions of Superoxide dismutase (Sod), Tumour necrosis Factor Receptor (Tnfr), p53 protein, and progesterone receptor (Pgr) genes using brain tissues from Sprague Dawley rats.

MATERIALS AND METHODS

Twenty Sprague-Dawley albino rats (156.68 ± 4.72g), comprising 10 males and 10 females were collected from the Laboratory Animal Centre of the College of Medicine, University of Lagos, Nigeria. Animals were fed rat chow and distilled water *ad-libitum*. The animals were kept in metallic cages and maintained under standard animal house conditions, with a 12hour light/dark cycle, and temperature of 25±2°C. They had free access to rat chow and water *ad libitum*. Care of all animal was in accordance with the national law on animal care and use (Zimmermon,1983). 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.

Table 1: Grouping of animals

Group	No of animals	Treatment
Group 1	3	Control male rats administered distilled water
Group 2	3	Male Rats administered 5ml/Kg body weight orange juice
Group 3	3	Male rats administered 5ml/kg body weight vitamin C Solution
Group 4	3	Control Female Rats administered distilled water
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.

RNA Extraction/cDNA synthesis: After the seventh day of 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 500 ng oligo dT, 1X Script buffer, 0.1 mM DTT, 1U/μl Rnase inhibitor, 0.4 mM dNTP, 4.0 U/ul Reverse transcriptase enzyme. PCR conditions was described in the Jena Bioscience Reverse transcriptase kit.

Primer Design: mRNA sequences of the following genes Sod, Tnfr, P53, Pr, Esr and Actb (Internal Control) 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

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Gene	Accession Number	Primer	Sequence (5^1-3^1)
Estrogen Receptor	NM_012689.1	Esr fwd	5^1-GCT ATG GAA TCT GCC AAG GA-3^1
Estrogen Receptor		Esr rvs	5^1-GGC AGC TCT TCC TCC TGT TT-3^1
Progesterone Receptor	NM_022847.1	Pr fwd	5^1-AAG GAA GAT TCC CGC TTC TC-3^1
Progesterone Receptor		Pr rvs	5^1-GCC CTC GTA ACT TTC GTC TT-3^1
Tumour Necrosis Factor Receptor	NM_013091.1	Tnfr fwd	5^1-GTG CCT ACC CCA GAT TGA GA-3^1
Tumour Necrosis Factor Receptor		Tnfr rvs	5^1-CTT CAA GCT CCC CCT CTT TT-3^1
P53 Protein Gene	NM_030989.3	P53 fwd	5^1-GCG CAC AGA GGA AGA GAA TC-3^1
P53 Protein Gene		P53 rvs	5^1-CAA GGC CTC ATT CAG CTC TC-3^1
Beta Actin	NM_031144.2	Actb fwd	5^1-GGC ATG GGT CAG AAG GAT TC-3^1
Beta Actin		Act b rvs	5^1- ACA TGA TCT GGG TCA TCT TCT C-3^1

Relative gene quantitation: Polymerase chain reaction (PCR) was performed in a 25 μl reaction volume containing 5 μg of cDNA, 0.2mM 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. Thermal cycling was done using the Applied Biosystems Real Time PCR at 94°C for 2 minutes; 94°C for 30 seconds; 56°C for 30 seconds; 72°C for 30 seconds; and 72°C for 2 minutes. Step 2 to 4 was repeated 35

times and data was acquired using the Applied Biosystem SDS Software. Dissociation curve analysis was done in-order to check for unspecific amplifications. Data were analyzed using Graphpad Prism 5.0.

RESULTS

The results for the modulation of the Tnfr gene, Sod3 gene, p53 gene and estrogen receptor gene in both male and female rats used for this study, can be explained as follows.

In the male rats, orange juice produced a large fold change difference in the level of expression of Tnfr mRNA while vitamin C produces no fold change difference in the level of the Tnfr gene. However, in the female rats the vitamin C produce a very high fold change difference when compared with orange juice and the control group. The fold change difference observed in the mRNA level of the male rats between the control, vitamin C and the orange juice is very small (less than 0.5). In the female rats, orange juice administration results in high fold change difference when compared with the vitamin C and the control group (Figure 1a).

In both male and female rats, the orange juice produced a high fold change difference when compared with vitamin C and the control group. Vitamin C produced a small fold change difference in the expression of the p53 gene. The mRNA level of the estrogen receptor shows a progressive increase from the rats administered vitamin C to the rats administered orange juice (Figure 2a).

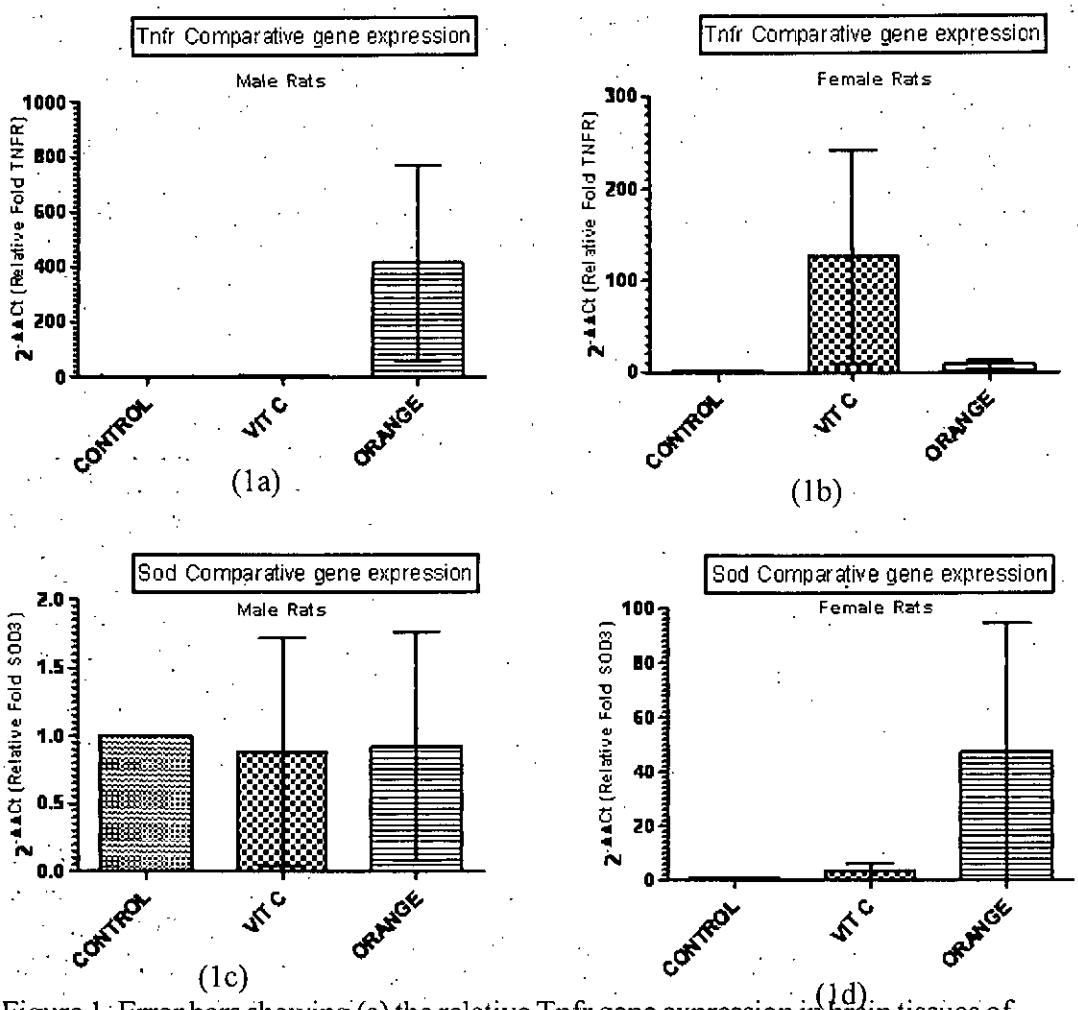

Figure 1. Error bars showing (a) the relative Tnfr gene expression in brain tissues of male rats, (b) in brain tissues of female rats , (c) relative Sod gene expression in brain tissues of male rats (d) in brain tissues of female rats as measured by qPCR. Measurement is in relative fold change $2^{-\ddot{A}\ddot{A}Ct}$.

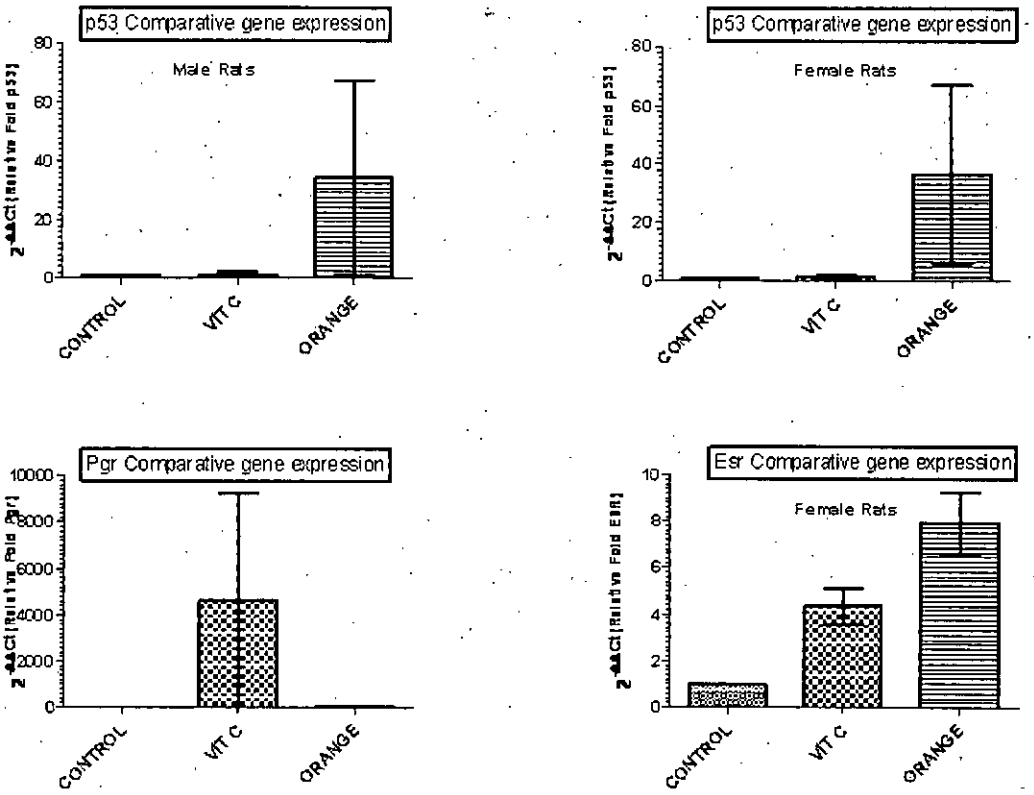

Figure 2. Error bars showing- (a) relative Tnfr gene expression in brain tissues of male rats (b) in brain tissues of female rats. (c) relative Sod gene expression in brain tissues of male rats (d) Esr gene expression in brain tissues of female rats as measured by qPCR. Measurement is in relative fold change $2^{-\Delta\Delta Ct}$.

DISCUSSION

Recent studies 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 increase in extracellular nitric oxide syntase (eNOS) expression (De-Nigris *et al.*, 2005). Red grape juice selectively reduced p47phox, p22phox and gp91phox expression at both mRNA and protein levels (Davalos *et al.*, 2009). Orange, which is a citrus fruit, and one of the most abundant and affordable fruits consumed by every population across the whole world. Orange juice has been shown to prevent oxidative stress and modulate the expression of genes (Husam *et al.*, 2010, Fatemah *et al.*, 2009). This present study revealed that orange juice and its component vitamin C can modulate the expression of genes. The administration of orange juice

results in an increase in the mRNA expression level of p53 and Tnfr gene. These two genes are involved in apoptotic processes. The p53 gene fold change difference observed in the male and female rats were very similar. The ability of vitamin C to produce a significant fold change in the Tnfr gene of the female rats indicate that the vitamin might be responsible for some of the observed changes in the male gene expression. The fold change migorrecctedht have occurred as a result some other bioactive components that are present in the orange juice, which can affect the expression of genes. These active components may be flavonoids and even some mineral elements that are present in the orange juice. For example, the major flavonoid in orange juice, hesperidine, decreases the expression of multidrug resistance associated protein in rat's small intestine and liver (Minoru *et al.*, 2011). Furthermore, minerals such as zinc, magnesium in the form of magnesium sulphate, and calcium have been shown to modulate the expression of genes. Blanchard *et al.* (2001) revealed that dietary zinc can modulate intestinal gene expression. They observed that most of the genes affected by dietary zinc participate in redox responsive pathway which may dysregulate as a consequence of a shift in cellular redox during zinc deficiency. Ca^{2+} -dependent regulation of neuronal gene expression have been reported (Bito *et al.*, 1997). In another research, magnesium sulphate has been shown to alter cerebellar gene expression responses to hypoxia (Haramati *et al.*, 2009).

The increase in the expression of p53 and Tnfr promote apoptosis. Manipulation of the apoptotic functions of these genes constitutes an attractive target for cancer therapy (Jiang *et al.*, 2007). The ability of orange juice to increase the expression of these genes indicates that it may have potential preventive effects of cancer. However, in neurodegenerative disorders, apoptosis results in cell death and poses deleterious effect on the body, increased consumption of orange juice will promote the expression of the p53 and Tnfr genes which may promote the severity of the disorder.

Orange juice produced a noticeable fold change in the female rats when compared with vitamin C and the control. However, both orange juice and vitamin C resulted in a small change in fold difference in the Sod3 mRNA expression level in the male rats, that is the level of SOD3 gene expression is transiently affected by vitamin C and orange juice. 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. The SOD1, SOD2, and SOD3 are members of the antioxidant enzyme superoxide dismutase family that catalyses the spontaneous dismutation of superoxide anion to hydrogen peroxide and oxygen. These findings suggest that the SOD genes response to fruit juice is transient.

The Esr gene showed a progressive increase in fold change from rats administered vitamin C to rats administered orange juice. This finding suggests that vitamin C is responsible for some of the alteration in the mRNA

expression level produced by the orange juice. This result indicates that vitamin C can modulate gene expression. The ability of vitamin C to alter gene expression has been demonstrated by various research studies. Passage *et al.,(2004)* demonstrated that treatment of a mouse model of the Charcot-Marie-Tooth 1A disease reverts, at least partly, to the transgenic mouse phenotype. This disease is due to the over-expression of a major myelin gene, *PMP22*, and ascorbic acids treatment lowers *PMP22* expression. In the same year, using microarrays representing about 6000 genes, Shin *et al.* (2004) published a series of genes responding to ascorbic acids treatment of embryonic stem cells. Most of the overexpressed genes belonged to gene families involved in neurogenesis, maturation and neurotransmission. Park *et al.*, (2009) published a proteomic analysis of cancer cells treated with ascorbic acids. The most relevant effects seem to be over-expression of RKIP and Annexin A5. Belin *et al.*, (2009) published an article describing changes of gene expression in normal and cancer cells treated with increasing doses of ascorbic acids.

CONCLUSION

In this study, orange juice showed the ability to increase mRNA expression level of the p53 and Tnfr genes that promote apoptosis. The increase in the expression of these genes may be mediated by other bioactive components in the orange juice rather than vitamin C, which will further scientific research. These findings may have implications in the management of cancer. However, vitamin C increased the mRNA expression level of Esr gene suggesting that this vitamin can also affect the expression of some genes.

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