

Orange Juice or Fructose Intake Does Not Induce Oxidative and Inflammatory Response

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Husam Ghanim, PhD
Priya Mohanty, MD
Ram Pathak, MD
Ajay Chaudhuri, MD
Chang Ling Sia, BSc
Paresh Dandona, MD, PhD

Division of Endocrinology, Diabetes and Metabolism
State University of New York at Buffalo and Kaleida Health
3 Gates Circle
Buffalo, NY 14209

Running title: Orange juice does not induce oxidative stress and inflammation

Correspondence To:
Paresh Dandona, B.Sc., M.B. B.S., D.Phil., F.R.C.P.
Director, Diabetes-Endocrinology Center of Western NY
Chief of Endocrinology, State University of New York at Buffalo
3 Gates Circle
Buffalo, NY 14209
E-mail: pdandona@KaleidaHealth.org

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Abstract

Background

We have previously shown that 300 kcal from glucose intake induces a significant increase in reactive oxygen species (ROS) generation and nuclear factor κ -B (NF κ B) binding in the circulating mononuclear cells (MNC) in healthy normal subjects.

Hypothesis

We have now hypothesized that the intake of 300 Calories as (1.) orange juice or (2.) fructose, the other major carbohydrate in orange juice, would induce a significantly smaller response than that of glucose.

Investigation

Four groups (8 subjects each) of normal weight subjects were given a 300 Calories drink of glucose (75g), or fructose (75g) or orange juice or water sweetened with saccharin (control group) to drink and blood samples collected. There was a significant increase in ROS generation by MNC (by $130\pm 18\%$, $P < 0.001$) and PMN (by $95\pm 22\%$, $P < 0.01$) and in NF κ B binding in MNC by $82\pm 16\%$ ($P < 0.01$) over the baseline after 2 hours of glucose intake. These changes were absent following fructose, orange juice or water intake. There was a significantly lower ROS generation and NF κ B binding following orange juice, fructose and water when compared with glucose ($P < 0.001$ for all). Furthermore, incubation of MNC, in vitro, with 50mM of the flavonoids hesperetin or naringenin reduced ROS generation by $52\pm 7\%$ and $77\pm 8\%$ ($P < 0.01$), respectively, while fructose or ascorbic acid did not cause any change.

Conclusion

Caloric intake in the form of orange juice or fructose does not induce either oxidative or inflammatory stress, possibly due to its flavonoids content, and might, therefore, represent a potentially safe energy source.

Our previous work has shown that the intake of glucose (75g= 300 kcalories, cream (33g= 300 kcalories) and a fast food meal (900 kcalories) induce an increase in reactive oxygen species (ROS) generation by peripheral blood mononuclear cells (MNC) in parallel with an increase in intranuclear nuclear factor κ B (NF κ B) DNA binding, a decrease in inhibitor κ B β (I κ B β) with an increase in I κ B kinase- α IKK α and IKK β expression and IKK activity (1-3). Intravenous infusion of glucose also results in an increase in pro-inflammatory cytokines, if endogenous insulin secretion is inhibited concomitantly with somatostatin (4). Glucose intake also results in an increase in pro-inflammatory transcription factors, AP-1 and Egr-1 and the corresponding genes activated by them: MMP-2, MMP-9, tissue factor and plasminogen activator inhibitor-1 (PAI-1) in normal subjects (5) and in vitro (6;7)

This raises the question whether caloric intake in any form induces oxidative stress and inflammation and whether the type of response is determined by the source of these calories. This is important since obesity, the result of excessive macronutrient intake, is characterized by an excess of oxidative stress and inflammation (8-10). Furthermore, the restriction of caloric intake in obese subjects results in a reduction of oxidative stress (8;9) and inflammatory mediators. A 48 hour fast in normal subjects results in a 35% reduction in ROS generation in 24 hours and > 50% reduction in 48h (11). Clearly therefore, caloric intake is a major source of oxidative and inflammatory stress. Since atherosclerosis, the major cause of cardiovascular death, is associated with oxidative stress and inflammation in the arterial wall (12), the search for foods that

are least likely to cause oxidative stress and inflammation, must be pursued.

Citrus juices, especially orange juice have been recommended by several health and nutrition groups as a healthy source of calories and their intake is associated with improved lipid profile and a reduced risk of cardiovascular disease (13;14). Furthermore, orange juice is a rich source of flavonoids and vitamin C (15;16) which may suppress ROS generation and inflammatory processes. It is also possible that flavonoids contained in orange juice may reduce or prevent oxidative stress and inflammation induced by macronutrients like glucose, fructose and sucrose contained in it.

Therefore, we hypothesized that orange juice intake induces less oxidative stress and inflammation than an equicaloric amount of glucose.

Methods

Subjects. Four groups, 8 each, of healthy normal weight subjects (Body Mass Index (BMI) 20-25 kg/m²) ages 20-40 yrs were recruited for this study. Three groups were given a single 300 kcal challenge of glucose, fructose or orange juice while subjects from the fourth group were given only water sweetened with saccharin. All subjects were given 10 minutes to finish their drinks. Fasting blood sample was collected before and at 1, 2 and 3 hr following the drink intake. An IRB approved consent form was obtained from all subjects.

MNC isolation: Blood samples were collected in Na-EDTA as an anticoagulant. Three and a half mL of anti-coagulated blood sample are carefully layered over 3.5 mL of Lympholyte medium (Cedarlane

Laboratories, Hornby, ON) and centrifuged to separate the cells. A top band consists of MNC and a bottom consists of polymorph nuclear cells (PMN) are collected. This method provides yields greater than 95% pure PMN and MNC suspensions.

ROS generation measurement by chemiluminescence: Five hundred μL of PMN or MNC (2×10^5 cells) are delivered into a Chronolog Lumiaggregometer cuvette. Luminol was then added, followed by 1.0 μL of 10 mM formylmethionyl leucyl phenylalanine (fMLP). In this assay system, the release of superoxide radical as measured by chemiluminescence has been shown to be linearly correlated with that measured by the ferricytochrome C method. The interassay coefficient of variation of this assay is 8 %. We have further established that the biological variation in reactive oxygen species generation in normal subjects is approximately 6 % for readings obtained 1 to 2 weeks apart.

NF κ B DNA binding activity: Nuclear NF κ B DNA binding activity was measured by electrophoretic mobility shift assay (EMSA). Nuclear extract were prepared from MNC and by high salt extraction. The specificity of the bands was confirmed by supershifting these bands with specific antibodies against Rel-A (p65) and p50 (Santa Cruz Biotechnology, CA) and by competition with cold oligonucleotides.

Measurement of Plasma glucose, insulin and CRP concentrations. Glucose levels were measured in plasma by YSI 2300 STAT Plus glucose analyzer (Yellow Springs, Ohio) and insulin was measured by an enzyme-linked immunosorbent assay kit (Diagnostics Systems

Laboratories, Inc., Webster, TX). Serum CRP was measured using an ELISA kit from Alpha Diagnostic International (San Antonio, TX).

ROS generation by MNC in vitro. Freshly isolated MNC from fasting normal subjects were incubated for 1 hr in PBS containing 5 mM glucose with either fructose (5 mM), vitamin C (0.250 mM ascorbic acid) or the flavonoids, hesperetin (50 μM) and naringenin (50 μM) dissolved in dimethyl sulfoxide (DMSO). Cells were washed once and ROS assay was carried as previously described. Controls included cells incubated with glucose with or without DMSO. This experiment was repeated 4 times and data represents the mean \pm SE. Inhibition of ROS generation by MNC was evaluated by incubating freshly isolated MNC with the NADPH oxidase inhibitor DPI (Sigma, San Antonio, TX) at concentration 10-1250 nM for 30 min. Cells were washed once and ROS generation measured.

Statistical analysis. Statistical analysis was conducted using SigmaStat software (SPSS Inc., Chicago, IL). All the data are represented as mean \pm S.E. Statistical Analysis from baselines was carried out using Holm-Sidak one-way repeated measures analysis of variance (RMANOVA). Dunnett's two-factor ANOVA method was used for all multiple comparisons between different groups/students t test for impaired data was used to compare Δ glucose/ Δ insulin ratio 1 hour after glucose or orange juice intake. ROS generation from the in vitro experiment was compared between the treatments using paired t-test analysis.

Results

Effect of the different treatments on plasma glucose and insulin concentrations. Plasma glucose concentrations increased from a mean of 94±6mg/dl to 129±21, 109±20 and 94±10mg/dl at 1, 2 and 3 h respectively after glucose intake and from 90±4mg/dl to 116±9, 102±5 and 92±5mg/dl at 1, 2 and 3 h respectively following orange juice (P<0.001 RMANOVA for both, Fig.1, On line appendix: <http://care.adajournals.org>). There was no significant difference in glucose values between glucose and orange juice intake. There was no significant change in glucose concentration following fructose or water intake. Plasma insulin concentrations increased from 9.5±2.44µU/ml to 47.0±8.3, 20.5±8.3 and 13.6±5.6 µU/ml at 1, 2 and 3 h respectively following glucose and from 6.1±1.8µU/ml to 50.3±13.1, 19.6±12.4 and 4.3±1.5 µU/ml at 1, 2 and 3 hr respectively following orange juice (P<0.001 RMANOVA for both, Fig. 2, On line appendix: <http://care.adajournals.org>). Fructose intake increased insulin concentrations from 8.8±1.3µU/ml to 17.0±5.2, 13.1±3.9 and 8.9±1.8 µU/ml at 1, 2 and 3 h respectively (P<0.01 RMANOVA). There was no difference in the change in insulin values following glucose and orange juice intake when compared by 2-factor RMANOVA but when compared to fructose, changes in insulin concentrations following both glucose and orange juice, were significantly greater (P<0.05). There was a trend towards a greater insulin concentration following orange juice for a given glucose concentration when compared to glucose intake. This was best expressed as a ratio between the increase in insulin and glucose concentrations at 1h. This ratio was significantly greater following orange juice

(Δ insulin/ Δ glucose ratio at 1 hr=1.77±0.32 vs.1.12±0.25; P=0.033, unpaired t-test).

Effect of the different treatments on ROS generation by MNC and PMN. There was a significant increase in ROS generation by MNC and PMN by 130±18% (P<0.001 RMANOVA, Fig. 1A) and 95±22% (P<0.01, RMANOVA, Fig. 1B), respectively, over the baseline within 2 hours of glucose intake. There was no significant change in ROS generation by MNC or PMN following fructose, orange juice or water intake. When ROS generation was compared between the groups, there was a significant difference between glucose intake and all of orange juice, fructose and water intake (P<0.01 for all, 2-factor RMANOVA). There was no significant difference in ROS generation between orange juice, fructose intake and water intake.

Effect of the different treatments on NFκB DNA binding in the MNC. NFκB DNA binding was increased significantly by 82±16% over the baseline (P<0.01 RMANOVA, Fig. 2) within 2 hours of glucose intake. There was no significant increase in NFκB binding following fructose, orange juice or water intake. When NFκB DNA binding changes were compared between the groups, there was a significant difference in NFκB binding following glucose intake when compared to baseline and to orange juice, fructose and water intake (P<0.01, for all, 2-factor RMANOVA). There was no significant difference in NFκB binding between orange juice, fructose intake and water intake.

Effect of 300 kcal intake on plasma CRP concentration. Although none of the challenges induced a significant change in

plasma concentrations of CRP over the 3 hours observation period, there was a trend towards a fall ($P < 0.1$, RMANOVA), and a significant fall in CRP concentrations within one hour of orange juice intake (1305 ± 375 vs. 1219 ± 321 ng/ml, $P = 0.044$ Paired t-test, Fig. 3). The change in CRP concentrations following orange juice was also significantly different when compared to water intake ($P < 0.01$, 2-factor RMANOVA).

Effect of orange juice components on ROS generation by MNC, in vitro. ROS generation by MNC freshly isolated from normal subjects was measured individually following 1 hr incubation with various components or orange juice. There was no significant change in ROS generation by MNC following incubation with 5mM fructose or 0.25 mM ascorbic acid when compared to control cells incubated with glucose alone. ROS generation was significantly lower when MNC were incubated with hesperetin (by $52 \pm 7\%$, $P = 0.004$) or naringenin (by $77 \pm 8\%$, $P = 0.002$), the 2 major flavonoids in orange juice, when compared to control cells incubated with glucose and DMSO alone (Fig. 4).

Effect of NADPH inhibition on ROS generation by MNC, in vitro. There was a significant dose dependent inhibition of ROS generation by MNC following 30 min incubation in 5mM glucose with increasing concentrations (10-1250 nM) of the NADPH oxidase inhibitor DPI in DMSO (Table 1, On line appendix: <http://care.adajournals.org>) when compared to control cells incubated with glucose and DMSO alone. This experiment was repeated 3 times on MNC from 3 different normal subjects.

Discussion

Our data confirm that the intake of 75g glucose induces a significant increase in ROS generation and NF κ B binding by MNC at 1 and 2 h after the intake. In contrast, equivalent amounts (300 Calories) of either orange juice or fructose did not induce a change in either ROS generation or NF κ B binding. These data are important since they show for the first time that 'safe' nutritional choices on the basis of minimizing post prandial oxidative and inflammatory stress can be made. It is relevant that alcohol intake (300 Calories) also does not result in increased ROS generation or NF κ B binding as reported by us previously (17). However, alcohol cannot and should not be recommended as a major macronutrient source.

We have previously shown that the diphenylene iodonium (DPI), a specific inhibitor of NADPH oxidase (18), completely inhibits ROS generation by leucocytes in a dose dependent manner (19). Our repeat experiments confirm this as presented here. This indicates that in our assay system ROS generation is mainly due to the activation of the NADPH oxidase which is located mainly in the leukocyte membrane in order to assist in bacterial killing following phagocytosis. Further testing with specific inhibitors of the mitochondrial electron transport chain is needed to totally exclude possible contribution of the mitochondria to ROS generation in our assay system. However, the fact that DPI dose dependently inhibits the ROS generation in a cell based assay system like ours implies that the majority of ROS detected in our system are the products of NADPH oxidase. Supporting this concept we have previously shown that superoxide dismutase also markedly suppresses ROS generation in this system.

It is intriguing that the absence of an increase in ROS generation and NFκB binding following orange juice was observed in spite of an increase in plasma glucose concentration which was not significantly different from that observed following glucose. This raised the question whether the presence of flavonoids and vitamin C exerted an ROS and NFκB binding suppressive effect or that fructose, the other major sugar in orange juice, may be suppressive of ROS generation and NFκB binding. Therefore we tested these possibilities in a separate series of experiments conducted, *in vitro*. We were able to demonstrate that fructose and ascorbic acid did not suppress ROS generation. While hesperetin and naringenin inhibited ROS generation by 52% and 77%, respectively, at micromolar concentrations. Clearly, therefore, the two major flavonoids in orange juice might mediate the suppression of glucose induced ROS generation. This area requires further investigation including work, *in vivo*.

Since fructose is a major sugar in fruits and other vegetable products, it would be worth investigating the potential anti-inflammatory effect of food products rich in fructose. It is also of interest that 75g fructose did not alter plasma glucose concentrations significantly while it did induce a small but significant increase in plasma insulin concentrations at 1h. It is possible that fructose is taken up and metabolized by the β cell resulting in a small release of insulin in fashion similar to that observed with glucose. The absence of oxidative and inflammatory stress following fructose is intriguing since fructose diets in animals induce insulin resistance. However, the quantities of fructose contained in high fructose

diets are far greater than those contained in a 300 calorie drink of orange juice.

It is appropriate to state that all calories in orange juice are from sugars: glucose (30%), fructose (30%) and sucrose (40%) (20). Sucrose is hydrolyzed in the gastrointestinal tract by disaccharidases into an equal number of glucose and fructose molecules. Thus, for all practical purposes, orange juice has glucose and fructose as the two major sugars in equal amounts. It is therefore, intriguing that, although the average increase in glucose and insulin concentrations following orange juice are not significantly different from those following glucose, the average increase in insulin concentrations calculated as $\Delta\text{insulin}/\Delta\text{glucose}$ ratio at 1h for each subject was significantly higher following orange juice intake. This suggests that orange juice may be more insulinogenic compared to glucose and that changes in glucose concentrations might not always totally predict plasma insulin response. Similar observations have been reported following the intake of orange juice and other fruit juices in terms of plasma glucose and insulin responses in type 2 diabetics (21). A higher insulin response may contribute, independently from the flavonoids effect, to the absence of oxidative stress and inflammation following orange juice since insulin reduces ROS generation, NFκB activation and CRP concentrations in humans *in vivo* (22;23).

The data on the effect of orange juice on plasma CRP concentrations are interesting. Although orange juice did not induce a significant fall in CRP over the observation period of 3 hours, there was a trend ($P=0.1$ RMANOVA) towards a reduction and a significant fall at 1 hour ($P<0.05$ by paired t-test) from baseline.

There was also a highly significant ($P < 0.01$, 2-factor RMANOVA) difference between the effect of water and orange juice on CRP concentrations following their intake. The clinical significance of a fall of CRP by 10-15% for a period of 2-3 hours following orange juice is not clear. Studies on the long term effect of orange juice intake in high risk groups are necessary to evaluate the clinical implications of a fall in CRP.

Our data are relevant to patients with diabetes since oxidative stress (24) and inflammatory (25) stress are markedly increased in this condition and may contribute to accelerated atherosclerosis. Clearly, the choice of foods which either do not increase or actually decrease oxidative and inflammatory stress in

diabetic subjects is important. Our findings also raise the issue of whether hypoglycemia in diabetics should be treated with glucose or orange juice. Further studies are required.

In conclusion, (1.) orange juice or fructose taken in equicaloric amounts to 75g of glucose does not cause either oxidative stress or inflammation in contrast to glucose; (2.) the two flavonoids, hesperetin and naringenin, have a ROS suppressive effect, in vitro, which needs to be confirmed, in vivo; (3.) ascorbic acid does not exert this effect; and (4) there are ways of avoiding post prandial oxidative stress and inflammation by making appropriate choices. The search for safe non-inflammatory foods and diets must continue.

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Figures Legends:

Fig. 1: ROS generation by MNC (A) and PMN (B) following glucose, fructose orange juice and water ingestion. *=P<0.01 RMANOVA for glucose; \$ P<0.01 2-factor RMANOVA between glucose and all other groups, n=8 for all.

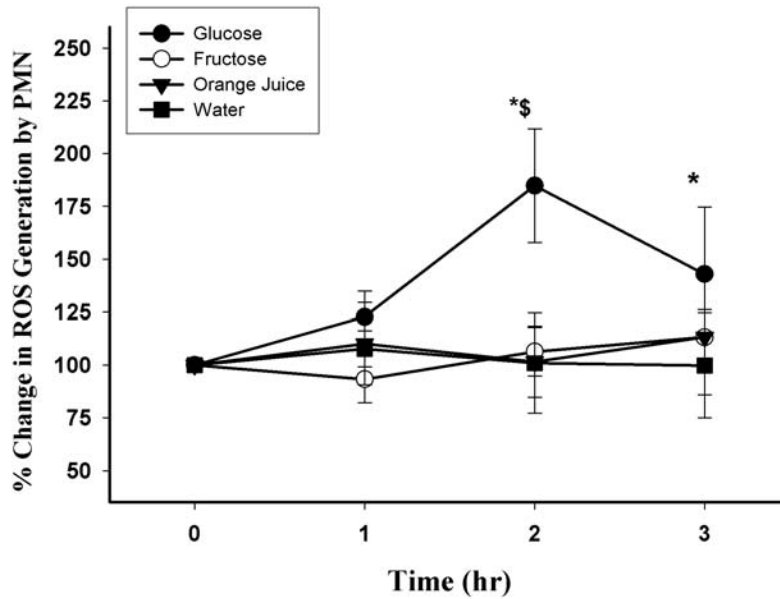
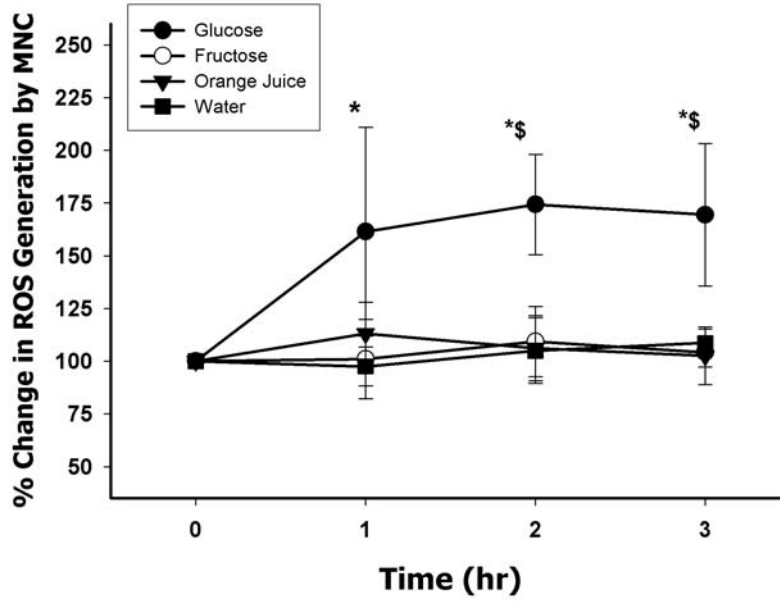


Fig. 2: Change in intranuclear NFκB binding activity following glucose, fructose orange juice and water ingestion. *=P<0.01 RMANOVA for glucose; \$=P<0.01 2-factor RMANOVA between glucose and all other groups, n=8 for all.

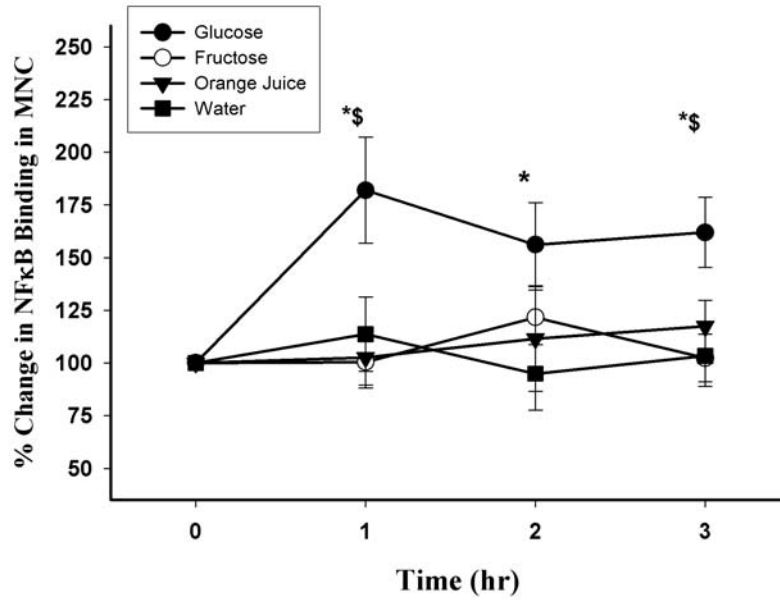


Fig. 3: Change in plasma CRP concentrations following glucose, fructose orange juice and water ingestion. #= $P < 0.05$ Paired t-test for orange juice; \$ $P < 0.01$ 2-factor RMANOVA between orange juice and water, $n=8$ for all.

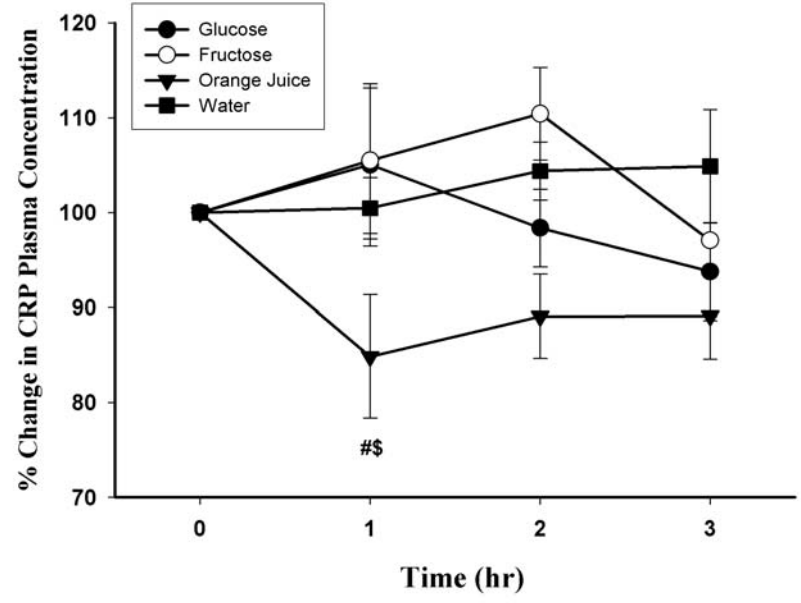


Fig. 4: ROS generation by MNC in vitro following 1 hr incubation with orange juice components (fructose (5 mM), vitamin C (0.250 mM ascorbic acid), hesperetin (50 μ M) naringenin (50 μ M). Control cells were incubated for 1 hr with 5 mM glucose alone or with DMSO. *=P<0.01, Paired t-test; n=4 and data represents the mean \pm SE.

