Journal of Horticulture
ISSN: 2376-0354

Saleh et al., J Hortic 2017, 4:3
DOI: 10.4172/2376-0354.1000209

Research Article Open Access

Atherogenicity of Monosaccharides, Disaccharides and Artificial Sweeteners in the Lipid-Laden Macrophage Model System: Cell Culture and Mice Studies

Saleh NA[1], Hamoud S[2], Aviram M[1]*, Rom O[1], Volkova N[1] and Hayek T[1,2]

[1]*The Lipid Research Laboratory, Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel*
[2]*Department of Internal Medicine E, Rambam Health Care Campus, Haifa, Israel*

Abstract

Background: Glucose is known to enhance macrophage foam cell formation and atherosclerosis development. However, the role of other monosaccharides, disaccharides or artificial sweeteners in macrophage atherogenicity remains unclear.

Objective: We thus compared their effects on oxidative status, cholesterol, and triglycerides accumulation which regulate foam cell formation.

Results: Supplementation of C57/BL6 mice for four weeks with sweeteners revealed that glucose, fructose, mannose, lactose or sucrose significantly increased hepatic lipid peroxidation and cholesterol accumulation, as well as mouse peritoneal macrophages (MPM) generation of ROS and lipid content. Supplementation with artificial sweeteners showed no significant pro-oxidative/atherogenic effects in the mice liver or aorta. Yet, cyclamate and sucralose significantly increased MPM ROS generation, and all artificial sweeteners increased MPM cholesterol content. In cultured J774A.1 macrophage cell line, glucose demonstrated the most pro-oxidative/atherogenic effects and significantly increased reactive oxygen species (ROS) generation (by 80%), cellular protein oxidation (by 119%), the accumulation of cholesterol and triglycerides (by 65% and 51%, respectively), and the macrophage phagocytosis capacity (by 177%). Mechanistically, glucose attenuated HDL-mediated cholesterol efflux from macrophages (by 17%) and enhanced their triglyceride biosynthesis rate (by 51%). Although to a lesser extent, mannose or cyclamate demonstrated pro-oxidative/atherogenic effects and significantly increased cellular ROS generation, cholesterol content, triglyceride content and macrophage phagocytosis capacity.

Conclusions: Taking together, the above results indicate the key pro-oxidative/atherogenic role for glucose as compared to other monosaccharides, as well as disaccharides or artificial sweeteners. Finally, the detrimental pro-atherogenic effects on macrophage foam cell formation of mannose or cyclamate, and to a lesser extent fructose, aspartame and saccharin are now clearly shown.

Keywords: Sugars; Sweeteners; Macrophage foam cells; Seahorse; Oxidative stress; Lipid metabolism; Atherosclerosis

Introduction

Atherosclerosis is the underlying cause of cardiovascular diseases (CVD), the major cause of death worldwide [1,2]. Atherosclerosis is an inflammatory disease of the arteries in which activated macrophages are abundant in the atherosclerotic lesions [3]. Macrophages and their oxidative status play key roles during early atherogenesis [3, 4]. After differentiating from peripheral monocytes, the formed intimal macrophages incorporate oxidized lipoproteins and are transformed into lipid-rich foam cells, the hallmark feature of early atherosclerosis [3]. In addition to lipoprotein uptake, lipid accumulation in macrophages can also result from alterations in cellular lipid metabolism, e.g. attenuated reverse cholesterol transport or enhanced rates of lipid biosynthesis; all are considerably affected by the oxidative status of the cells [4].

High intake of added sugars increases the risk of CVD and type 2 diabetes mellitus (T2DM) [5]. T2DM and hyperglycemia are associated with accelerated atherosclerosis mediated by enhanced macrophage foam cell formation [6]. Numerous studies have demonstrated the detrimental role of high glucose on macrophage oxidative status or lipid metabolism leading to foam cell formation. Accelerated atherosclerosis in diabetic mice was associated with macrophage lipid peroxidation and increased generation of reactive oxygen species (ROS) via induction of NADPH oxidase [7,8]. In addition, macrophages from diabetic mice or under high glucose conditions exhibit lipid accumulation mediated by various mechanisms that regulate intracellular lipid metabolism [7-15]. These include enhanced uptake of oxidized (ox)-LDL via up-regulation of the scavenger receptors CD36 and SR-A [7-9,11], enhanced cholesterol or triglyceride biosynthesis rates via induction of lipid biosynthesis regulators e.g. the sterol regulator elements binding proteins (SREBPs), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) or diacylglycerol acyltransferase1 (DGAT1) [12,14], and attenuation of HDL-mediated cholesterol efflux from macrophages via suppression of ATP-binding cassette (ABC) transporters ABCA1 and ABCG1 [10,13,15].

Although the pro-oxidative and pro-atherogenic role of glucose in macrophage foam cell formation has been established, little is

***Corresponding author:** Michael Aviram, The Lipid Research Laboratory, Rappaport Faculty of Medicine, Technion – Israel Institute of Technology, Israel, Tel: +972 4 8295278; Fax:+972 4 8520076; E-mail: aviram@tx.technion.ac.il

Received August 28, 2017; **Accepted** September 12, 2017; **Published** September 20, 2017

Citation: Saleh NA, Hamoud S, Aviram M, Rom O, Volkova N, et al. (2017) Atherogenicity of Monosaccharides, Disaccharides and Artificial Sweeteners in the Lipid-Laden Macrophage Model System: Cell Culture and Mice Studies. J Hortic 4: 209. doi: 10.4172/2376-0354.1000209

known about the effects of other monosaccharides or disaccharides on macrophage atherogenicity. Macrophages were shown to express the glucose transporter (GLUT) isoforms 1 and 3 that transport mainly glucose, but also galactose and mannose into the cells [16-19]. The expression of GLUT1 and GLUT3 by foam cells was suggested to promote their lipid loading [17]. Macrophages were also shown to express GLUT5 which catalyzes the transport of fructose into the cells, but the role of GLUT5 in foam cell formation remains unknown [17, 18]. Notably, in LDL receptor (LDLR)-deficient mice, a high-fructose diet was more atherogenic than a high-fat diet, promoting macrophage accumulation in the atherosclerotic lesions of the mice [19]. In primates, a high fat and high fructose diet resulted in aortic infiltration of lipid-laden foam cells [20]. As for disaccharides, it was demonstrated that macrophages are able to take up or hydrolyze various disaccharides including sucrose, lactose or maltose [21]. Nevertheless, the role of the above monosaccharides or disaccharides in macrophage foam cell formation is yet unknown.

In recent years, the use of artificial sweeteners as an alternative to added sugars has been significantly increased, and approximately 30% of adults and 15% of children reported using artificial sweeteners in the USA [22,23]. The commonly consumed artificial sweeteners include aspartame, saccharin, sucralose, and the dietary supplement stevia, an extract from the leaves of the *Stevia rebaudiana* plant [22,23]. Nevertheless, accumulating evidence suggests that increased consumption of these artificial sweeteners may be associated with increased risk of T2DM and CVD similarly to high sugar intake [23]. Several large-scale studies have demonstrated that consumption of artificially sweetened beverages is associated with increased risk for coronary heart disease (CHD) or other vascular events in a similar magnitude to consumption of sugar-sweetened beverages [22,24-26].

While previous studies have demonstrated a link between the consumption of artificial sweeteners and CVD risk, only a few mechanistic studies have focused on the effects of artificial sweeteners on atherosclerosis development, the underlying cause of CVD [24-26]. In atherosclerotic apolipoprotein E-deficient (apoE-/-) mice, consumption of aspartame-acesulfame K sweetened 'light' cola was found to accelerate the progression of atherosclerotic plaques and the accumulation of sub-endothelial lipid-laden macrophages [27,28]. Also, *in vitro* treatment of apoA-I or HDL with physiological concentrations of aspartame, acesulfame K, or saccharin resulted in their pro-oxidative and pro-atherogenic modifications [29,30]. On the other hand, consumption of stevioside by leptin and LDLR double knockout mice was reported to reduce the aortic plaque volume by decreasing the content of macrophages, lipids, and ox-LDL in the plaque [31].

The aim of the current study thus was to investigate and compare the *in vitro* and *in vivo* effects of various monosaccharides (glucose, fructose, galactose, or mannose), disaccharides (sucrose, lactose or maltose), and artificial sweeteners (aspartame, cyclamate, saccharin, sucralose or steviol) on macrophage oxidative status, lipid metabolism and the related mechanisms that regulate foam cell formation and enhanced atherogenesis.

Experimental Procedures

Materials

Monosaccharides (glucose, fructose, galactose, and mannose), disaccharides (lactose and sucrose), artificial sweeteners (saccharin, cyclamate, aspartame, sucralose and steviol), fluorescein-isothiocyanate (FITC), dimethylformamide (DMF), 5, 5-dithiobis-(2-nitrobenzoic acid, 2, 7-dichlorofluorescin diacetate (DCFH-DA), dihydrocumarin, paraoxon, chloramine-T (N-chloro-p-toluenesulfonamide sodium salt), seahorse medium, potassium iodide (KI) and the triglyceride determination kit (containing the T2449 triglyceride reagent, and the F6428 free glycerol reagent) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Phosphate buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM), DMEM without glucose, fetal calf serum (FCS), penicillin, streptomycin, nystatin, L-glutamine, sodium pyruvate, bovine serum albumin (BSA), enzyme-linked chemiluminescence (ECL) solution, and pHrodo® Green Zymosan Bioparticles® for phagocytosis were all purchased from Biological Industries (Beth Haemek, Israel). [^{3}H]-Labeled acetate and [^{3}H]-labeled oleic acid were purchased from PerkinElmer (Waltham, MA, USA). Silica gel plates (60F254) and lactate dehydrogenase (LDH) determination kit were purchased from Merck (Darmstadt, Germany). Cholesterol measurement kit (CHOL, 11491458), Accu-Chek glucose sensor and test strips and protease inhibitor cocktail tablets (cOmplete 11231400) were obtained from Roche Diagnostics (Mannheim, Germany). Rodent chow was purchased from Altromin (Lage, Germany). Bradford reagent and PVDF membrane were purchased from Bio-Rad (Hercules, CA, USA). RNA purification kit (MasterPure TM) was obtained from Epicentre Biotechnologies (Madison, Wisc., USA). cDNA preparation kit and ABsolute Blue qPCR ROX mix were purchased from Thermo Scientific (Epsom, UK). Primary antibody against HMGCR, DGAT-1, Nrf2, and PON2 were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Primary antibody against actin (MAB1501, mouse monoclonal antibody) was purchased from Millipore (Temecula, CA, USA). Horseradish peroxidase-conjugated secondary antibodies (AffiniPure Donkey Anti-Mouse, Goat Anti-Rabbit or Rabbit Anti-Goat polyclonal antibodies) were obtained from Jackson Immuno-Research (West Grove, PA, USA).

Mice study

The study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institute of Health, USA. The study protocol was approved by the Committee for Supervision of Animal Experiments of the Technion – Israel Institute of Technology (Approval number: IL-045-04-2016). The effects of glucose or artificial sweetener feeding were studied in C57BL/6 mice. This mice strain was chosen since it does not develop atherosclerosis spontaneously as genetically altered mouse models commonly used to study atherosclerosis such as apoE-/-or LDLR-/-mice, however, it is more susceptible to develop diet-induced atherosclerosis when compared to other strains such as C3H or BALB/c mice [32,33].

Monosaccharides, disaccharides or artificial sweetener feeding: Sixty-six male C57BL/6 mice aged 7 weeks were provided by Harlan Laboratories (Indianapolis, IN, USA). The mice were housed in pathogen-free conditions at the Animal Care Facility of the Faculty of Medicine, Technion. The mice were allowed 4 days of acclimatization period in which water and standard chow were available ad libitum. The doses of the artificial sweeteners were based on the acceptable daily intake (ADI) of the FDA and were set at 5 mg/kg/d for each one of the artificial sweeteners [34]. The dose of glucose was based on its relative sweetness when compared to the given dose of the artificial sweeteners (by 400-fold) and was set at 2000 mg/kg/d. Accordingly, the doses of the other monosaccharides and disaccharides was set at 2000 mg/kg/d. At 8 weeks of age, the mice were randomly

divided into the following experimental groups for a period of 4 weeks (n=6 per group): 1) Control: no supplementation, 2) glucose supplementation (2000 mg/kg/d), 3) fructose supplementation (2000 mg/kg/d), 4) mannose supplementation (2000 mg/kg/d), 5) lactose supplementation (2000 mg/kg/d), 6) sucrose supplementation (2000 mg/kg/d), 7) glucose supplementation (5 mg/kg/d – equivalent to the artificial sweetener doses), 8) saccharin supplementation (5 mg/kg/d), 9) cyclamate supplementation (5 mg/kg/d), 10) sucralose supplementation (5 mg/kg/d), 11) steviol (5 mg/kg/d). As the ADI for aspartame is 50 mg/kg/d, which is significantly higher than the ADI for the above artificial sweeteners [32], aspartame was not included in the mice study. The monosaccharides, disaccharides or the artificial sweeteners were administrated to the mice in their drinking water which were replaced every 3 days, and their complete ingestion by the mice was monitored and confirmed. Throughout the study, mice were allowed ad libitum access to chow and were weighed twice a week.

Isolation of mouse peritoneal macrophages (MPM): MPM (15-25 × 10^6 per mouse) were harvested from the peritoneal fluid of the anesthetized mice, three days after intraperitoneal injection of 3 ml of thioglycolate (24 g/L). MPM were washed with PBS then resuspended in DMEM containing 1000 U/L penicillin, 100 mg/L streptomycin and 5% heat-inactivated FCS and incubated in a humidified incubator (37°C, 5% CO2). All assays were performed within 36 h following the seeding of the MPM.

Serum analyses: Blood was collected from the retro-orbital plexus of mice under isoflurane anesthesia. Serum cholesterol, triglycerides and glucose were measured using commercially available kits as described in section 2.4. Levels of lipid peroxidation were measured by the lipid peroxides assay or TBARS assays [35,36]. Serum PON1 activity was determined spectrophotometrically at 412 nm with paraoxon as a substrate. The assay mixture included 10 µl of non-diluted serum, 4 mM paraoxon, 50 mM glycine and 1 mM CaCl2. One unit of paraoxonase activity= 1nmol of hydrolyzed paraoxon per min/1mL serum [37].

Aorta and liver analyses: Aortas and livers were removed from the euthanized mice and kept at -80°C. Then, tissue samples were homogenized in PBS using Polytron Homogenizer (Kinematica AG, Littau, Switzerland). Sample homogenates were then centrifuged (13,000 g, 15 min) and the supernatants were analyzed for protein levels by the Lowry assay [38]. Aortic or hepatic lipids were extracted with hexane:isopropanol (3:2, v:v), and the hexane phase was evaporated under nitrogen. Cholesterol and triglycerides were determined with commercially available kits as described in section 2.4., and were expressed as µg cholesterol or triglycerides/µg protein. Lipid peroxidation was measured by the lipid peroxides assay [36], and was expressed as nmol lipid peroxides/µg protein.

J774A.1 macrophages studies: J774A.1 murine macrophage-like cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in a humidified incubator (37°C, 5% CO2) in regular DMEM containing 25 mM glucose 1,000 U/L penicillin, 100 mg/L streptomycin and 5% heat-inactivated FCS. For monosaccharides and artificial sweetener experiments, macrophages (1 × 10^6) were incubated for 24 h with low glucose DMEM containing 5 mM glucose, 1,000 U/L penicillin, 100 mg/L streptomycin and 5% heat-inactivated FCS. The next day, macrophages were incubated for 18 h with DMEM without glucose (control) or with 5 mM of monosaccharides (glucose, fructose, galactose, or mannose) or artificial sweeteners (saccharin, cyclamate, aspartame, sucralose or steviol).

Macrophage analysis

Toxicity

The effects of each one of the monosaccharides and artificial sweetener on macrophage toxicity were assessed by determination of cellular protein concentration using the Lowry assay [38], by cell counting using a hemocytometer counting strategy, and also by the release of LDH into the medium as previously described [39].

XFe96 Real-time bioenergetic measurements (Glycolysis stress test)

The effects of 5 mM of each one of the monosaccharides, disaccharides or artificial sweetener on the glycolysis and respiratory oxidative phosphorylation of J774A.1 macrophages (3 × 10^4 cells) were determined using the Seahorse XF96 Extracellular Flux Analyzer (Seahorse Bioscience), as described in the manufacturer protocol. Rates of oxygen consumption (OCR; an indicator of oxidative phosphorylation) and extracellular acidification rate (ECAR; an indicator of glycolysis) were measured, in real-time. Cells were washed with XF Assay Media (un-buffered, glucose free, pyruvate free, medium with 2 mM L-glytamine), then 180 µl of medium were added to each well. Cells were treated first with 5 mM monosaccharides, disaccharides or artificial sweetener then with 1 µM oligomycine (inhibits Complex V; ATP synthase), followed by 50 mM of 2-deoxy-D-glucose (2-DG), a glycolysis inhibitor [40]. The remaining cells were let to dry, dissolved in 0.1 M NaOH, and cellular protein was measured using the Lowry assay [38].

Macrophage oxidative status

Macrophage ROS generation: The effects of each one of the monosaccharides, disaccharides or artificial sweetener on intracellular ROS generation in macrophages were determined with the DCFH-DA probe as previously described [41]. Briefly, following the treatments, the cells were washed with PBS and incubated for 40 min with 10 µM of DCFH-DA at 37°C in the dark. Then, the cells were washed with PBS, and the adherent cells were detached by gentle scraping. Measurements of cellular fluorescence were determined by flow cytometry and performed using BD LSRFortessa (BD Biosciences, San Jose, CA, USA). Results are expressed as percentage of control.

Macrophage protein oxidation: Advanced oxidation protein products (AOPP) assay was used to evaluate the level of macrophage protein oxidation as previously described [42]. Following the treatments, J774A.1 macrophages (1 × 106 cells) were washed with PBS and cellular protein was extracted with PBS and protease inhibitor. Chloramine-T stock solution was freshly prepared with PBS and used for the calibration curve (12.5, 25, 50, 75, and 100 mM). Potassium iodide (KI, 1.16 M) was also prepared in PBS. 200 µl of protein sample was added to a 96 well microplate, incubated for 25 s and then the absorbance of the mixture was read at 340 nm. Then, 20 µL of acetic acid was added, mixed and incubated for 25 s. Next, 10 µL of KI solution was added to the reaction mixture, mixed and after the elapse of additional 25 s the absorbance was read again at 340 nm. All steps were carried out at 37°C. Protein concentration was measured using Lowry assay [38]. Results are expressed as µM Chloramine-T equivalents/mg cell protein.

Macrophage paraoxonase2 (PON2) lactonase activity: Following the treatments, PON2 lactonase activity was measured using dihydrocumarin as the substrate [43]. Initial rates of hydrolysis were determined spectrophotometrically at 270 nm. The assay mixture included 1 mM dihydrocumarin solution and 1 mM CaCl2 in 50 mM

Tris-HCl, pH 8.0 and was added to the cells for 10 min. The absorbance was read at 270 nm against reaction buffer. Non-enzymatic hydrolysis of dihydrocumarin was subtracted from the total rate of hydrolysis. One unit of lactonase activity equaled to 1 μmol of dihydrocumarin hydrolyzed/min/ml. The remaining cells in the dish were washed and dissolved in

0.1 M NaOH, and cellular protein was measured using the Lowry assay [38]. Results are expressed as lactonase units/mg cell protein.

Macrophage total thiol content (SH groups): Following the treatments, macrophage total thiol content was measured which determines the amount of protein bound SH groups, as well as glutathione [44]. An aliquot of 10 μl cell lysate was mixed with 190 μl of Tris–EDTA buffer, pH 8.2, and the absorbance was measured at 412 nm. Then, 8 μl of 10 mM DTNB was added, and after 15 min incubation at room temperature the absorbance was measured, together with a DTNB blank. Total SH groups are calculated as previously described [44].

Macrophage Lipid metabolism

Macrophage cholesterol mass

Following the treatments, macrophages were washed twice with PBS and cellular lipids were extracted with hexane: isopropanol (3:2 v/v). The hexane phase was evaporated under nitrogen. The content of cellular cholesterol was determined as previously described [41], using the commercially available kit detailed in section 2.4. The remaining cells in the plates were dissolved in 0.1 M NaOH, and an aliquot was taken for the measurement of cellular protein by the Lowry assay [38]. Results are expressed as μg cholesterol/mg cell protein.

Macrophage triglyceride mass

Following the treatments, macrophages were washed twice with PBS and cellular lipids were extracted as described in section 2.4. The content of cellular triglycerides was determined as previously described [41], using the commercially available reagents detailed in section 2.4. The remaining cells in the plates were dissolved in 0.1 M NaOH, and an aliquot was taken for the measurement of cellular protein by the Lowry assay [38]. Results are expressed as μg triglycerides/mg cell protein.

Macrophage cholesterol or triglycerides biosynthesis rate

Following the treatments, macrophages were incubated with [^{3}H]-acetate (for cholesterol biosynthesis rate) or [^{3}H]-oleic acid (for triglycerides biosynthesis rate) for 3 h at 37°C, followed by cellular lipid extraction with hexane:isopropanol (3:2 v/v) and separation by thin-layer chromatography (TLC) on silica gel plates using a mixture of 130 ml hexane, 30 ml ether, and 1.5 ml acetic acid. The spots of unesterified cholesterol or triglycerides were visualized by iodine vapor, using an appropriate standard for identification, scraped into scintillation vials, and counted for radioactivity by β-counter (Packard Tri Carb 2100TR, PerkinElmer, Waltham, MA, USA). The remaining cells in the dish were dissolved in 0.1 M NaOH, and cellular protein was measured using the Lowry assay [38]. Results are expressed as cpm/mg cell protein.

Macrophage triglyceride hydrolysis rate

Macrophages were washed with PBS and further incubated for 3 h at 37 °C in serum-free medium containing

0.2% BSA and 3 μCi/mL [^{3}H]-oleate. Then, the cells were washed twice with PBS and incubated (0 h which reflects the results of the triglyceride biosynthesis assay, 1 and 4 h) with serum-free medium containing 0.2% BSA. At the end of the above incubation periods, the cells were washed with PBS, lipids were extracted and radiolabeled triglycerides were analyzed as described above. After extraction of cellular lipids, the cells were dissolved in 0.1 M NaOH for measurement of cellular protein by the Lowry assay [38]. Results are expressed as cpm/mg cell protein.

HDL-mediated cholesterol efflux from macrophages

Following the treatments, J774A.1 macrophages were incubated with [^{3}H]-labeled cholesterol (2 μCi/ml) in serum-free medium supplemented with 0.2% BSA for 1 h at 37°C. After washing with PBS (x3), the cells were further incubated with DMEM in the presence of HDL (100 μg HDL protein/mL, see section 2.4.4.6) or in the absence of HDL (basal, non-specific loss of cholesterol from the cells to the medium) for 3 h at 37 °C.

Cellular and medium [^{3}H]-labels were quantified by β-counter (Packard Tri Carb 2100TR, PerkinElmer, Waltham, MA, USA), and the basal or HDL-mediated cholesterol efflux was calculated as the ratio of [^{3}H]-label in the medium/([^{3}H]-label in the medium + [3H]-label in the cells). Net HDL-mediated cholesterol efflux data were corrected for the basal.

Lipoprotein isolation

Lipoproteins were isolated from fresh plasma derived from healthy subjects by discontinuous density gradient ultracentrifugation as previously described [45]. Briefly, for VLDL separation, saline-EDTA solution, density

1.006 g/ml, pH 7.4, was added to the plasma, then ultracentrifuged for 18 h at 4°C and the upper phase was collected. For LDL separation, the density of the plasma was raised to 1.063 g/ml with KBr, followed by ultracentrifugation for 24 h at 4°C and collection of the upper phase. For HDL separation, the density of the plasma was raised to 1.21 g/ml, followed by ultracentrifugation for 48 h at 4°C and collection of the upper phase.

Lipoprotein labelling

Lipoproteins (LDL and VLDL) were labelled with FITC as previously described [46]. Briefly, the lipoproteins (protein concentration for LDL was 1 mg/ml and 0.8 mg/mL for VLDL) were dialyzed overnight at 4°C against several changes of borate buffer containing 0.1 M borate, 25 mM sodium tetraborate, 75 mM NaCl, pH 8.6. 1 h prior to FITC conjugation, the pH of the dialysis buffer was altered to 9.4. FITC was dissolved in dimethyl formamide and added to the lipoproteins to give a final concentration of 0.2 mg/mL and then incubated for 1 h at room temperature with stirring in the dark. FITC-conjugated lipoproteins were separated from unconjugated FITC by size exclusion chromatography over a PD-10 column, eluting with 10 mM phosphate buffer at pH 8.0.

Macrophage uptake of LDL

Following the treatments, J774A.1 macrophages were incubated for 3 h at 37°C with LDL at a final concentration of 10 μg of protein/ml in serum free DMEM supplemented with 0.2% BSA. The uptake of FITC-conjugated LDL by the cells was determined by flow cytometry (BD LSRFortessa, BD Biosciences, San Jose, CA, USA) and the results were expressed as mean fluoresce intensity (MFI).

Macrophage uptake of triglyceride-rich VLDL

Following the treatments, the uptake of VLDL by the macrophages was determined as previously described [47]. Briefly, J774A.1

macrophages were incubated for 3 h at 37°C with 20 μg/mL of FITC-conjugated VLDL in DMEM without FCS, supplemented with 0.2% BSA. The uptake of FITC-conjugated VLDL by the cells was determined by flow cytometry (BD LSRFortessa, BD Biosciences, San Jose, CA, USA) and the results were expressed as MFI.

Macrophage phagocytosis of Zymosan bioparticles

The effects of each one of the monosaccharides or artificial sweetener on the uptake of zymosan bioparticles by J774A.1 macrophages (1×10^4 cells) were determined with the IncuCyte ZOOM Live-Cell Imaging System (Essen Bioscience, Ann Arbor, Michigan, USA) as described in the company protocol. Briefly, macrophages (1×10^4 cells) were seeded in 96-wall plate for 18 h, then the treatments (5 mM) were added with pHrodo Green Zymosan Bioparticles (sonicated for 15 min, final concentration of 10 μg/well). The plate was immediately placed in the IncuCyte ZOOM system, 10x objective scans were set to instantly start and scheduled for 24 h with repeated scans every 30 min to monitor fluorescence changes. Phagocytosis was quantified in the IncuCyteTM software by determining the intensity of green-fluorescent objects (phagosomes) in the field of view over time. Data are expressed as the intensity of objects per image.

Macrophage lysates and Western blot analysis

Macrophages were washed twice with PBS and lysed using RIPA lysis buffer. Total protein concentrations were measured by the Bradford assay, using BSA as a standard. A total protein of 30 μg/lane was loaded and separated by standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Following SDS-PAGE, proteins were transferred to PVDF membranes. Membranes were then blocked with 5% BSA in TBS-T (0.1% Tween) for 1 h and exposed overnight to primary antibody at 4°C. Primary antibodies were diluted 1:500 except for actin antibody which was diluted 1:4000. Next, membranes were washed with TBS-T followed by 1 h incubation at room temperature with appropriate secondary antibodies. Detection was performed with ECL using ImageQuant LAS 4000 digital imager system (GE Healthcare, Bucks, UK). Protein quantities were determined by densitometry and analyzed using Total Lab Software V2006C (Nonlinear Dynamics, Newcastle-on-Tyne, UK). Actin was used for normalization of protein quantities as a loading control. Results were expressed as relative protein levels vs. control.

Macrophage RNA extraction and quantitative PCR analysis

Total RNA was extracted with MasterPure TM RNA purification kit. cDNA was generated from 1 μg of total RNA with Verso TM cDNA kit. Using ABsolute Blue qPCR ROX mix, products of the reverse transcription were subjected to quantitative PCR using TaqMan gene expression assays with a Rotor-Gene 6000 amplification detection system. PON2, diacylglycerol acyltransferase1 (DGAT1), hydroxyl-methylglutaryl-CoA reductase (HMGCR) and Nuclear factor erythroid-derived 2 (Nrf2) mRNA data were normalized to GAPDH mRNA, used as an internal standard. All primers and probes were designed by PrimerDesign (South Hampton, UK). Results were expressed as relative mRNA levels vs. control.

Statistical analysis

All statistics were performed with Prism software (GraphPad Software, Inc, La Jolla, CA 92037 USA). Correlations were performed with SPSS (International Business Machines (IBM) Corp. New Orchard Road Armonk, New York), with pearson correlation coefficient. Results are presented as mean ± SEM of at least three independent observations. Statistical analysis was performed by one-way analysis of variance (ANOVA) for repeated measures followed by the Dunnett post-hoc test to compare the treatment effects with those of glucose treatment or the control. $p<0.05$ was considered statistically significant.

Results

The effects of oral supplementation of C57BL/6 mice with monosaccharides, disaccharides or artificial sweeteners

The effects of four weeks of supplementation of C57BL/6 mice with monosaccharides, disaccharides or artificial sweeteners were assessed. First, daily solution consumption, food consumption, and weekly mice body weight gain were evaluated. During the four-week supplementation period, there was no significant difference in solution consumption or food consumption between the different groups, as well; body weight gain was similar in all groups (Supplementary Table 1).

The effects of supplementing C57BL/6 mice with monosaccharides, disaccharides or artificial sweeteners on serum, liver, and aorta oxidative stress

The effects of supplementing C57BL/6 mice with monosaccharides, disaccharides or artificial sweeteners for four weeks on serum, liver and aorta were evaluated. Glucose (2000 mg/kg/day) significantly increased hepatic lipid peroxidation by 77% ($p<0.05$, Table 1), with a trend towards increased aortic lipid peroxidation (by 51%, $p>0.05$, Table 1). Supplementation of the mice with fructose, mannose, lactose, and sucrose (2000 mg/kg/day) increased the hepatic, but not the aortic, lipid peroxidation by 178% ($p<0.0001$), 65%, 71%, and 91% ($p<0.05$), respectively (Table 1). No significant effects were observed on lipid peroxidation in the serum, liver or aorta of the C57BL/6 mice receiving the artificial sweeteners supplementations (Table 1). Nevertheless, steviol significantly decreased serum PON1 lactonase activity by 38% ($P<0.0001$).

The effects of supplementing C57BL/6 mice with monosaccharides, disaccharides or artificial sweeteners on serum, liver, and aorta lipid metabolism

Supplementation of the mice with glucose (2000 mg/kg/day) had no significant effects on serum cholesterol or triglyceride levels. However hepatic cholesterol levels significantly increased by 100% ($p<0.05$) following glucose supplementation (Table 1). Fructose, mannose, lactose and sucrose had no significant effects on aorta or serum cholesterol levels, but increased hepatic cholesterol levels by 116% ($p<0.05$), 150% ($p<0.05$), 133% ($p<0.05$) and 200% ($p<0.01$), respectively (Table 1). The sweeteners sucralose and steviol showed a trend of increment in aortic cholesterol by 38% and 25%. Furthermore, lactose and steviol significantly increased serum triglyceride concentration by 36% ($p<0.01$) and 23% ($p<0.01$), respectively (Table 1).

The effects of supplementing C57BL/6 mice with monosaccharides, disaccharides or artificial sweeteners on MPM oxidative status

The effects of supplementing C57BL/6 mice with monosaccharides, disaccharides or artificial sweeteners on the generation of ROS in MPM were evaluated next. MPM from mice treated with glucose (2000 mg/kg/day) showed significantly higher ROS generation, as compared to control group (by 108%, $p<0.0001$, Figure 1A). Lower supplementation with glucose (5 mg/kg/day) had no significant effects on the oxidative status of the mice (Figure 1B). Similarly, MPM from mice treated with fructose, mannose, lactose, and sucrose for 4

	Serum						Liver			Aorta		
	Oxidation		Anti- Oxidation		Lipid metabolism		Oxidation	Lipid metabolism		Oxidation	Lipid metabolism	
	AAPH-induced TBARS (nmol /mL serum)	AAPH-induced lipid peroxidation (nmol /mL serum)	PON Arylesterase activity (U/mL)	PON lactonase activity (U/mL)	Serum triglyceride concentration (mg/dL)	Serum cholesterol concentration (mg/dL)	Lipid peroxidation (nmol PD/µg protein)	Triglyceride content (µg/µg protein)	Cholesterol content (µg/µg protein)	Lipid peroxidation (nmol PD/ µg protein)	Triglyceride content (µg/µg protein)	Cholesterol content (ng/µg protein)
Control	13.1 ± 0.4	1269 ± 24	379 ± 56	147.3 ± 5	37.4 ± 2	51.8 ± 2	329 ± 24	1.12 ± 0.05	0.06 ± 0.006	250 ± 18	0.25 ± 0.03	8 ± 0.8
Glucose 5 mg/kg	13.9 ± 0.8	1235 ± 55	358 ± 10	165 ± 7	45 ± 0.7	55 ± 2	290 ± 15	0.99 ± 0.04	0.05 ± 0.004	371 ± 22	0.27 ± 0.03	8.6 ± 1.4
Glucose 2 g/Kg	14.2 ± 0.6	1276 ± 11	366 ± 87	157.4 ± 4	46.7 ± 3	46.6 ± 1	583 ± 30 *	1.08 ± 0.03	0.12 ± 0.018 *	377 ± 61	0.27 ± 0.02	8 ± 0. 6
Fructose 2 g/Kg	14.5 ± 0.3	1359 ± 12	357 ± 47	176.2 ± 3 ***	38.4 ± 2	52 ± 1	915 ± 64 ***	1.09 ± 0.06	0.13 ± 0.015 *	212 ± 11	0.26 ± 0.02	7 ± 1.1
Mannose 2 g/Kg	14.3 ± 0.4	1331 ± 15	382 ± 48	176.3 ± 3 ***	45 ± 2	50 ± 1	542 ± 60	1.07 ± 0.06	0.15 ± 0.017 *	279 ± 20	0.28 ± 0.02	7 ± 0.7
Lactose 2 g/Kg	13.4 ± 0.8	1354 ± 29	378 ± 41	178 ± 2 ***	51 ± 3 **	48.4 ± 1	562 ± 90	1.20 ± 0.06	0.14 ± 0.023 *	281 ± 36	0.23 ± 0.03	8 ± 1.7
Sucrose 2 g/Kg	10.9 ± 0.3	1350 ± 16	301 ± 38	176 ± 4 ***	35 ± 2	45 ± 2 *	629 ± 74 *	1.21 ± 0.07	0.18 ± 0.024 **	229 ± 28	0.28 ± 0.04	7 ± 0.7
Saccharin 5 mg/kg	13.7 ± 0.4	1312 ± 18	349 ± 14	162 ± 6	42 ± 0.5	51 ± 2	305 ± 30	1.2 ± 0.23	0.05 ± 0.011	294 ± 27	0.24 ± 0.02	8 ± 1.6
Cyclamate 5 mg/kg	12.3 ± 1.0	1338 ± 20	321 ± 9	154 ± 7	35 ± 1.0	50 ± 4	360 ± 56	1.2 ± 0.15	0.06 ± 0.003	295 ± 24	0.24 ± 0.05	9 ± 2.3
Sucralose 5 mg/kg	12.2 ± 0.6	1309 ± 8	338 ± 11	154 ±	39 ± 0.6	49 ± 2	347 ± 13	1.1 ± 0.07	0.06 ± 0.003	358 ± 47	0.35 ± 0.03	11 ± 4.0
Steviol 5 mg/kg	12.5 ± 0.5	1346 ± 10	303 ± 16	92 ± 3 ***	46 ± 0.6 **	50 ± 2	344 ± 20	1.3 ± 0.06	0.07 ± 0.006	224 ± 19	0.33 ± 0.02	10 ± 1.3

The effects of oral supplementation of C57BL/6 mice with monosaccharides, disaccharides or artificial sweeteners on serum, liver, and aorta oxidative stress, cholesterol concentration, and triglyceride concentration. Fructose, mannose, lactose and sucrose decreased the serum PON1 antioxidant activity, and increased hepatic lipid peroxidation and cholesterol accumulation. Steviol treated mice demonstrated a significant decrease in serum PON activity, and increase in serum triglycerides. (*) P<0.05, (**) P<0.01, (***) P<0.0001 vs. control group (n=6)

Table 1: Serum, aorta and liver oxidation and lipid metabolism level, after four weeks of monosaccharides, disaccharides or artificial sweeteners supplementation in C57Bl/6 mice.

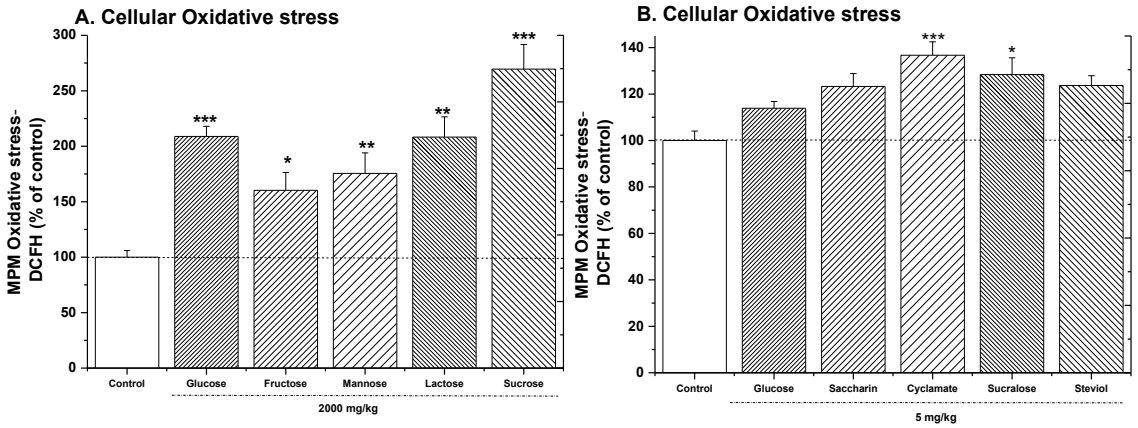

Figure 1: Effects of monosaccharides, disaccharides or artificial sweeteners on oxidative status in MPM from C57BL/6 mice: (A) MPM ROS Generation following four weeks of 2 gr/kg/d monosaccharides or disaccharides supplementation; (B) 5 mg/kg/d Glucose or artificial sweeteners supplementation were examined. (*) P<0.05; (**) P<0.01; (***) P<0.0001 vs. control group.

Weeks showed significant increase in ROS generation by 60% (p<0.05), 75% (p<0.01), 108% (p<0.01) and 170% (p<0.0001), respectively (Figure 1A). Cyclamate and sucralose significantly increased MPMs ROS generation by 37% (p<0.0001) and 28% (p<0.05), respectively, as compared to the control group (Figure 1B).

The effects of supplementing C57BL/6 mice with monosaccharides, disaccharides or artificial sweeteners on MPM lipid metabolism

Supplementation of the mice with glucose (2000 mg/kg/day) increased MPM cholesterol mass (by 102%, P<0.05, Figure 2A) which could be attributed to a significant increment in cholesterol biosynthesis rate (by 103%, P<0.05, Figure 2B) and attenuation of HDL-mediated cholesterol efflux from the MPM (by 28%, p<0.05, Figure 2C). Mannose demonstrated a significant increase in the MPM cholesterol mass (by 89%, p<0.05, Figure 2A), mainly as a result of increased LDL uptake (by 71%, p<0.05, Figure 2D). Fructose, lactose and sucrose showed similar trends of increment in MPM cholesterol metabolism as glucose (2000 mg/kg/day) (by 59%, 76% and 51%, Figure 2A). As to the artificial sweeteners; saccharin, sucralose and steviol significantly increased MPM cholesterol mass by 62% (P<0.01), 74% (P<0.01) and 83% (p<0.001) (Figure 2E). The MPM cholesterol content

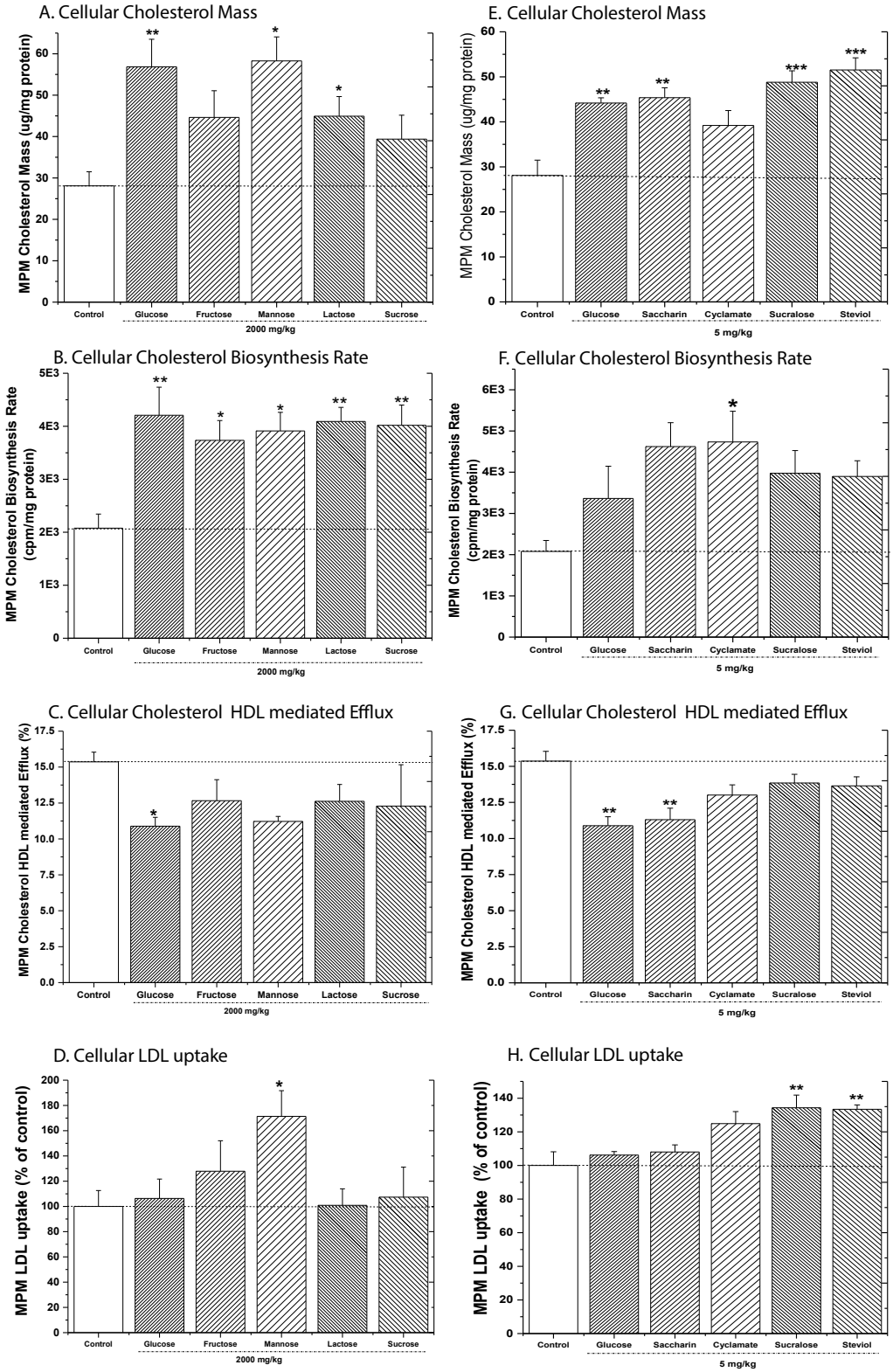

Figure 2: Effects of monosaccharides, disaccharides or artificial sweeteners on cholesterol metabolism in MPM from C57BL/6 mice: (A) Effects of four weeks of 2 gr/kg/d monosaccharides or disaccharides supplementation on MPM cellular cholesterol mass; (B) Cellular cholesterol biosynthesis rate cellular; (C) Cholesterol HDL-mediated efflux; (D) Cellular LDL uptake; (E) The effects of four weeks of 5 mg/kg/d glucose or artificial sweeteners supplementation on MPM cellular cholesterol mass; (F) Cellular cholesterol biosynthesis rate; (G) Cellular cholesterol HDL-mediated efflux and (H) Cellular LDL uptake were evaluated; (*) P<0.05; (**) P<0.01; (***) P<0.0001 vs. control group.

augmentation following treatment with saccharin may be attributed to the reduction in HDL-mediated cholesterol efflux (by 26%, P<0.05, Figure G). However, the increase in MPM LDL uptake may explain the increase in cholesterol content in the MPMs from sucralose and steviol treated mice (by 34% and 33%, p<0.01, Figure 2H).

As for MPM triglyceride metabolism, glucose supplementation (2000 mg/kg/day) increased MPM triglycerides content (by 138%, P<0.05, Figure 3A), triglycerides biosynthesis rate (by 67%, P<0.05, Figure 3B) and VLDL uptake (by 26%, P>0.05, Figure 3C). Sucrose showed significant increment in the MPM triglycerides content (by 145%, P<0.05, Figure 3A), without affecting triglycerides biosynthesis rate or VLDL uptake (Figures 3B and 3C). As compared to control group, neither low glucose (5 mg/kg/day) treated C57BL/6 mice nor the artificial sweeteners supplementation to mice showed any effects on MPMs triglycerides metabolism (Figure 3D).

Effects of monosaccharides, disaccharides or artificial sweeteners on J774A.1 macrophage

Bioenergetic profile of J774A.1 macrophages supplemented with monosaccharides, disaccharides or artificial sweeteners: The effects of 5 mM of glucose, other monosaccharides, disaccharides or artificial sweeteners on macrophage glycolysis and oxidative phosphorylation were determined next using Seahorse Extracellular Flux Analyzer. Acidification was rapid with glucose as a substrate, then increased, as expected, when mitochondrial ATP synthesis was inhibited by oligomycin and demolished by 2-DG (Figure 4A). The glycolysis process in glucose-treated macrophages increased by 169%, as compared to control group (P<0.001, Figure 4B). Mannose (Figures 4A and 4B) and Sucrose (Figures 4C and 4D) showed a slight increase in extracellular acidification rate by 65% and 55% respectively (P<0.05). No effects on glycolysis were observed following treatment with fructose, galactose, lactose, maltose or any of the artificial sweeteners (Figures 4A-4F). Oxygen consumption rate was not significantly different between the treatments (data not shown).

Effects of monosaccharides or artificial sweeteners on J774A.1 macrophage: The effects of 5 mM of glucose, other monosaccharides or artificial sweeteners on cultured macrophage cell line (J774A.1) oxidative status, lipid metabolism and phagocytosis were assessed next. The J774A.1 macrophages were not treated with disaccharides, since disaccharides are completely hydrolyzed to their monosaccharides; glucose, fructose, and galactose in the digestive system, and macrophages are not likely to interact with them. The selected concentration of monosaccharides or artificial sweeteners was not toxic to the cells. Cell toxicity was evaluated using protein content, cell count and LDH levels as indicators (data not shown).

Effects of monosaccharides or artificial sweeteners on J774A.1 macrophage oxidative status: As shown in Figure 5, glucose had a marked stimulatory effect on J774A.1 macrophage oxidative status. Compared to control cells, glucose significantly increased the generation of ROS (by 80%, p<0.0001, Figure 5A) and the formation of AOPP (by 119%, p<0.05, Figure 5B). In parallel, glucose significantly decreased PON2 activity (by 44%, p<0.0001, Figure 5C), as well as the cellular total thiols content (by 58%, p<0.01, Figure 5D). The monosaccharides mannose, fructose and galactose increased macrophage ROS generation by 64% (p<0.0001), 63% (p<0.0001), and 29% (P<0.05), respectively (Figure 5A). However, minor effects on cellular protein oxidation were observed following incubation of the cells with 5 mM mannose, fructose or galactose.

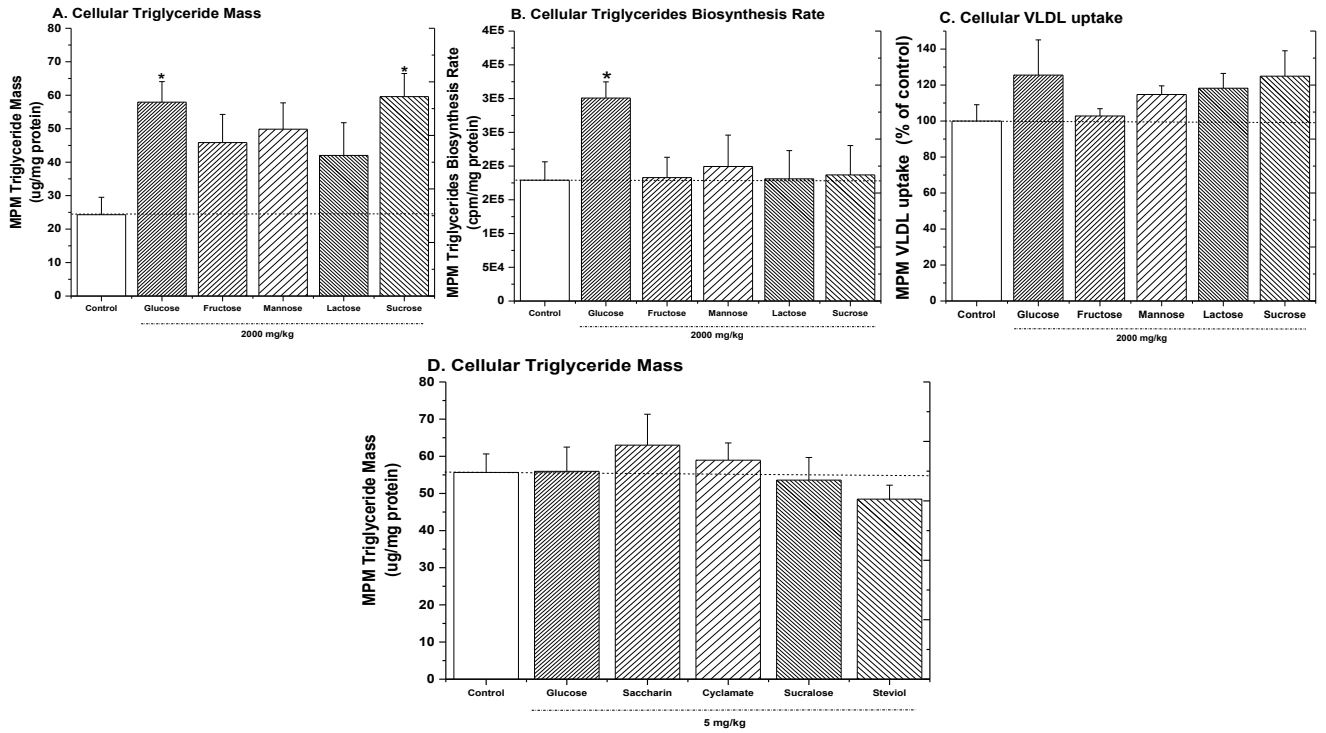

Figure 3: Effects of monosaccharides, disaccharides or artificial sweeteners on triglyceride metabolism in MPM from C57BL/6 mice: Effects of four weeks of 2 gr/kg/d monosaccharides, or disaccharides supplementation on MPM; (A) Cellular triglyceride mass; (B) Cellular triglyceride biosynthesis rate; (C) Cellular VLDL uptake in addition to effects of four weeks of 5 mg/kg/d glucose or artificial sweeteners supplementation on MPM; (D) Cellular triglyceride mass were examined. (*) P<0.05; (**) P<0.01; (***) P<0.0001 vs. control group.

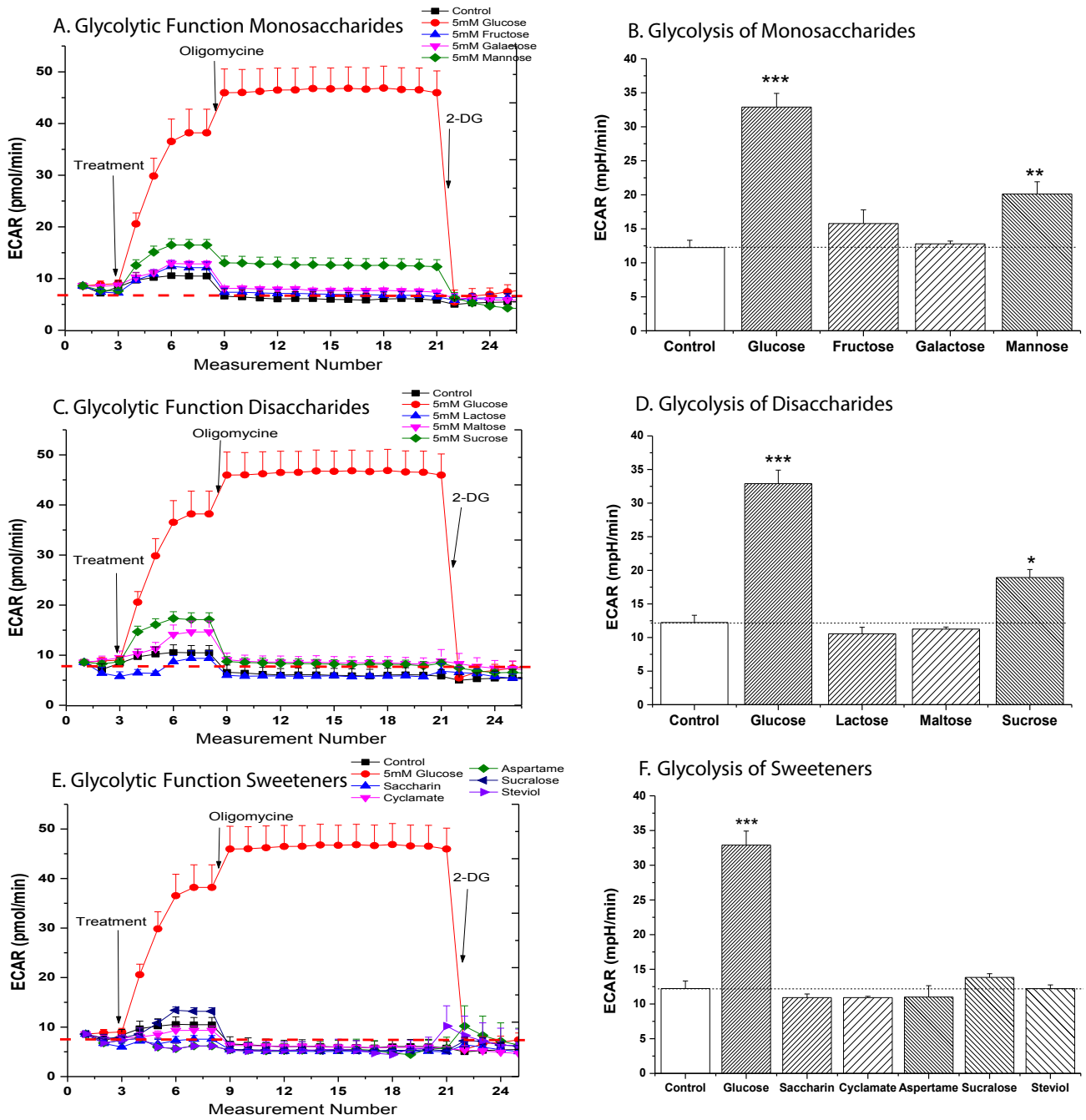

Figure 4: Effects of monosaccharides, disaccharides or artificial sweeteners on the Bioenergetic profile of J774A.1 macrophage cells: (A, B) Real-time evaluation of monosaccharides; (C, D) Disaccharides or artificial sweeteners; (E, F) Effects on the extracellular acidification rate (ECAR), an indicator of glycolysis. (*) P<0.05; (**) P<0.01; (***) P<0.0001 vs. control group.

(Figure 5B). In addition, mannose and fructose significantly decreased PON2 activity by 35% (p<0.01) and 28% (p<0.0001), respectively (Figure 5C), with trends toward decreased total thiols (Figure 5D).

As to the artificial sweeteners, cyclamate showed a similar pro-oxidative effect as glucose and significantly increased the generation of ROS (by 59%, p<0.0001, Figure 5E) and the formation of AOPP (by 166%, p<0.05, Figure 5F). Cyclamate also showed trends towards decreased PON2 activity (by 32%, p>0.05, Figure 5G) and total thiol content (by 43%, p>0.05, Figure 5H). Moreover, aspartame was found to significantly increase the generation of ROS (by 27%, p<0.05, Figure 5E) and to decrease PON2 activity (by 33%, p<0.05, Figure 5F), with trends towards higher AOPP formation (by 47%, p>0.05, Figure 5G) as well as lower total thiol content (by 36%, p>0.05, Figure 5H). Apart from cyclamate, all of the examined artificial sweeteners showed a significantly lower effect on macrophage ROS generation when compared with glucose (p<0.0001, Figure 5F), however no artificial sweetener demonstrated an anti-oxidative effect.

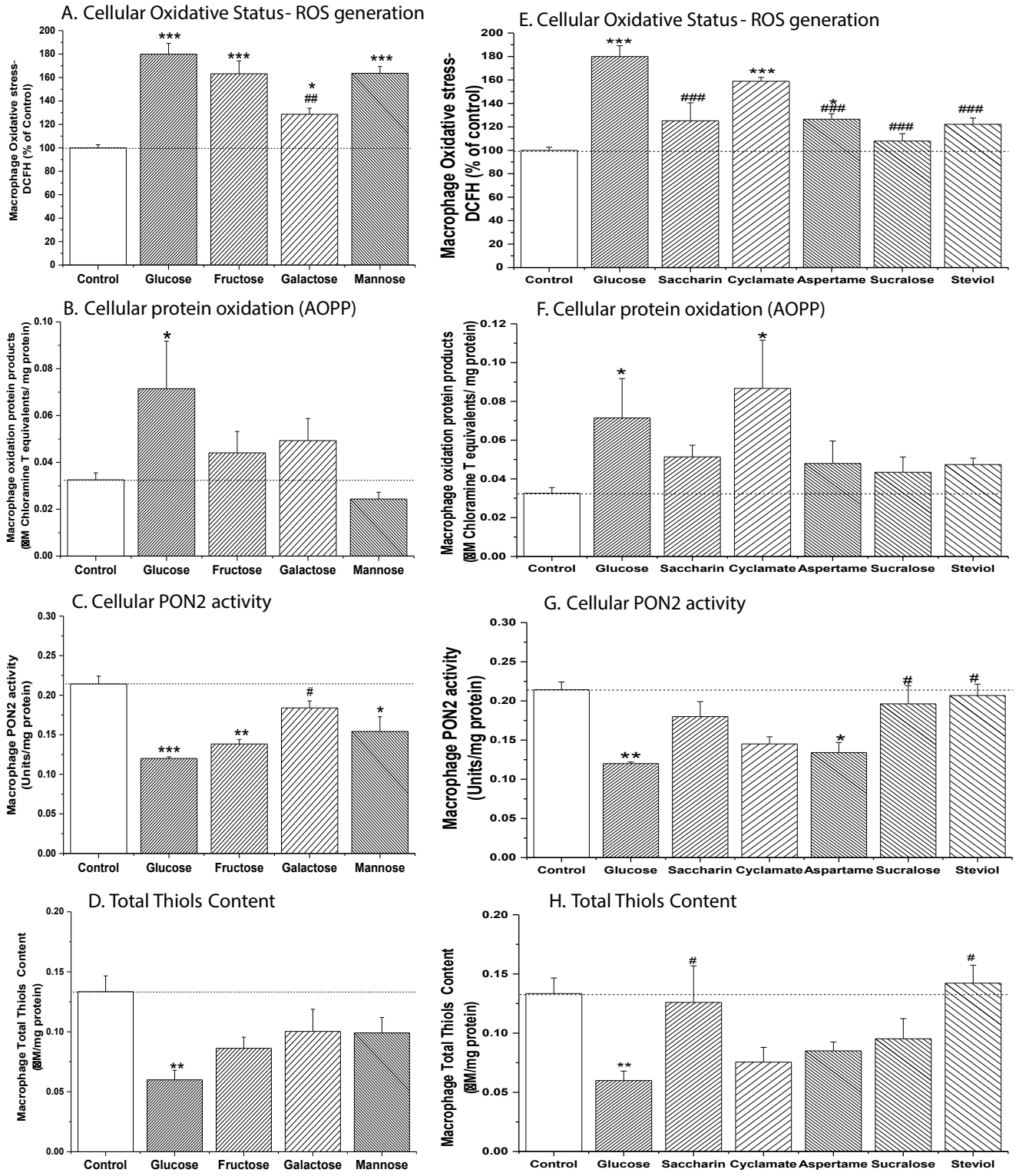

Figure 5: Effects of monosaccharides or artificial sweeteners on J774A.1 macrophage oxidative status: The effects of each one of the monosaccharides on the macrophage oxidative status after 18 h of incubation were assessed; (A) ROS generation by the DCFH-DA assay; (B) AOPP-Protein oxidation; (C)PON2 activity; and (D) Total thiol content. The effects of each one of the artificial sweeteners on the macrophage oxidative stress after 18 h of incubation were as well assessed; (E) DCFH-ROS generation; (F) AOPP-Protein oxidation; (G) PON2 activity; (H) Total thiol content; (*) P<0.05; (**) P<0.01; (***) P<0.0001 vs. control group; (#) P<0.05; (##) P<0.01; (###) P<0.0001 vs. glucose.

Effects of monosaccharides, or artificial sweeteners on J774A.1 macrophage cholesterol metabolism: The effects of glucose or other monosaccharides on macrophage cholesterol mass and metabolism (i.e. LDL-cholesterol uptake by macrophage, cellular cholesterol biosynthesis rate and HDL-mediated cholesterol efflux from the cells) was determined next. Glucose showed the most prominent effect and significantly increased macrophage cholesterol mass (by 65%, $p<0.0001$, Figure 6A), which could be attributed to a significant reduction in HDL-mediated cholesterol efflux from the macrophages (by 16% $p<0.05$, Figure 6C) and a trend towards enhanced cholesterol biosynthesis rate (by 42%, $p>0.05$, Figure 6B). Although to a lesser extent than glucose, mannose also significantly increased macrophage cholesterol mass (by 34%, $p<0.0001$, Figure 6A) which could be attributed to a significant increase in cholesterol biosynthesis rate (150% increase, $p<0.05$, Figure 6B). Fructose and galactose had no significant effects on macrophage cholesterol metabolism (Figures 6A-6C). All of the examined monosaccharides showed a significantly lower effect on macrophage cholesterol accumulation when compared with glucose ($p<0.0001$, Figure 6A). Compared with glucose, cellular cholesterol mass was significantly lower after treating the macrophages with each one of the examined artificial sweeteners ($p<0.0001$, Figure 6D), however, saccharin and cyclamate were found to significantly increase macrophage cholesterol mass when compared to control cells (by 28% and 23%, respectively, $p<0.0001$, Figure 6D). The effects of saccharin and cyclamate on cellular cholesterol mass could be attributed to increased LDL uptake by macrophages (by 28%, and 11%, $p<0.0001$, respectively, Figure 6G), with trends towards enhanced cholesterol biosynthesis rate (by 42% and 13%, respectively $p>0.05$, Figure 6E).

Taking together, these data indicate the pro-atherogenic effect of glucose on macrophage cholesterol accumulation which was found to a lesser extent for mannose, saccharin or cyclamate and was not evident following other treatments.

Effects of monosaccharides, or artificial sweeteners on J774A.1 macrophage triglyceride metabolism: The effects of glucose, other monosaccharides, or artificial sweeteners on macrophage triglyceride mass and metabolism (i.e. cellular triglyceride biosynthesis or hydrolysis rate) were determined next. In accordance with its stimulatory effects on macrophage oxidative status and cholesterol accumulation, glucose significantly increased macrophage triglyceride mass (by 51%, $p<0.0001$, Figure 7A), which could be attributed to a significant increase in cellular triglyceride biosynthesis rate (by 52% $p<0.0001$, Figure 7B). Mannose had similar effects as glucose and increased the cellular triglyceride mass by 48% ($p<0.0001$, Figure 7A). The increase in triglyceride mass following mannose treatment was not conveyed with significant increases in triglyceride biosynthesis rate (increased by 15%, $P>0.05$, Figure 7B) nor with attenuation in the triglyceride degradation rate (Figure 7C). Fructose and galactose had no significant impact on triglyceride mass or their biosynthesis rate (Figures 7A and 7B). Compared with glucose, cellular triglyceride mass was significantly lower after treating the macrophages with the artificial sweeteners cyclamate ($p<0.05$), sucralose ($p<0.01$) or steviol ($p<0.0001$), but when compared with control cells, macrophage triglyceride mass was significantly increased after treating the cells with saccharin (by 26%, $p<0.05$) or with aspartame (by 27%, $p<0.01$, Figure 7D). Unlike glucose, none of the artificial sweeteners enhanced the cellular triglyceride biosynthesis rate (Figure 7E). The impact of both saccharin and aspartame on macrophage triglyceride mass could be attributed to a significant attenuation of triglyceride hydrolysis rate (by 81% and 33%, respectively, $p<0.0001$, Figure 7F).

Altogether, unlike glucose which stimulated a marked triglyceride accumulation in macrophages via enhanced rate of triglyceride biosynthesis, none of the other monosaccharides or artificial sweeteners affected the triglyceride biosynthesis rate. Mannose, saccharin, and aspartame increased triglyceride content by attenuating the triglyceride hydrolysis rate.

Effects of monosaccharides or artificial sweetener on J774A.1 macrophage phagocytosis capacity: The effect of glucose, other monosaccharides or the artificial sweeteners on cellular phagocytosis were determined in the J774A.1 macrophage. The cells were incubated with the above treatments for up to 24 h, and cellular phagocytosis level was assessed in 30 min intervals by measuring the uptake of zymosan bioparticles. The phagocytosis level at 18 h of incubation was analyzed and presented in Figure 8.

From all of the examined treatments, glucose had the most prominent effect and was found to enhance the phagocytosis level by 177% as compared to control cells ($p<0.0001$, Figures 8A and 8B). The treatments associated with enhanced cholesterol mass and ROS generations were also accompanied with augmentation of the phagocytosis capacity (presented in Figures 8A and 8B). Mannose increased the phagocytosis level by 68% ($p<0.05$), and the artificial sweeteners saccharin, cyclamate and aspartame increased the phagocytosis level by 153%, 100% and 132%, respectively ($p<0.0001$, Figure 8B). Pearson correlation assessments were performed between the parameters of macrophage oxidative status, lipid metabolism and phagocytosis capacity for all monosaccharides and artificial sweeteners. The results revealed that the cellular cholesterol mass was positively correlated with the phagocytosis capability ($p=0.05$, Figure 8C). In addition, PON2 activity was significantly and negatively correlated with the phagocytosis level ($p<0.05$, Figure 8D). No correlation was found between cellular triglyceride content and phagocytosis, and the correlation with ROS generation was insignificant.

Taking together, glucose showed the most stimulatory effect on the phagocytosis capability, which was positively correlated with cellular cholesterol content and negatively correlated with PON2 lactonase activity. The summarized different impacts of monosaccharaides, disaccharides and artificial sweeteners on macrophage atherogenicity are presented in (Figure 9)

Discussion

In the current study, the effects of monosaccharides (glucose, fructose, galactose, or mannose), disaccharides (sucrose, or lactose), and artificial sweeteners (aspartame, cyclamate, saccharin, sucralose or steviol) on oxidative status, cholesterol, and triglycerides accumulation and foam cell formation (the hallmark of early atherogenesis), were compared for the first time. In C57BL/6 mice, supplementation with glucose, fructose, mannose, lactose, sucrose or cyclamate, all significantly increased hepatic lipid peroxidation and cholesterol accumulation as well as macrophage oxidative stress. Glucose showed the most prominent pro-oxidative and pro-atherogenic effect, but additional monosaccharides (mannose) or artificial sweeteners (cyclamate) were also found to increase macrophage oxidative stress and the accumulation of cholesterol or triglycerides, though to a lesser extent.

Atherosclerosis development and cardiovascular diseases are significantly affected by nutritional factors. Although much progress has been made in understanding the role of glucose and diabetes in macrophage foam-cell formation and atherosclerosis development

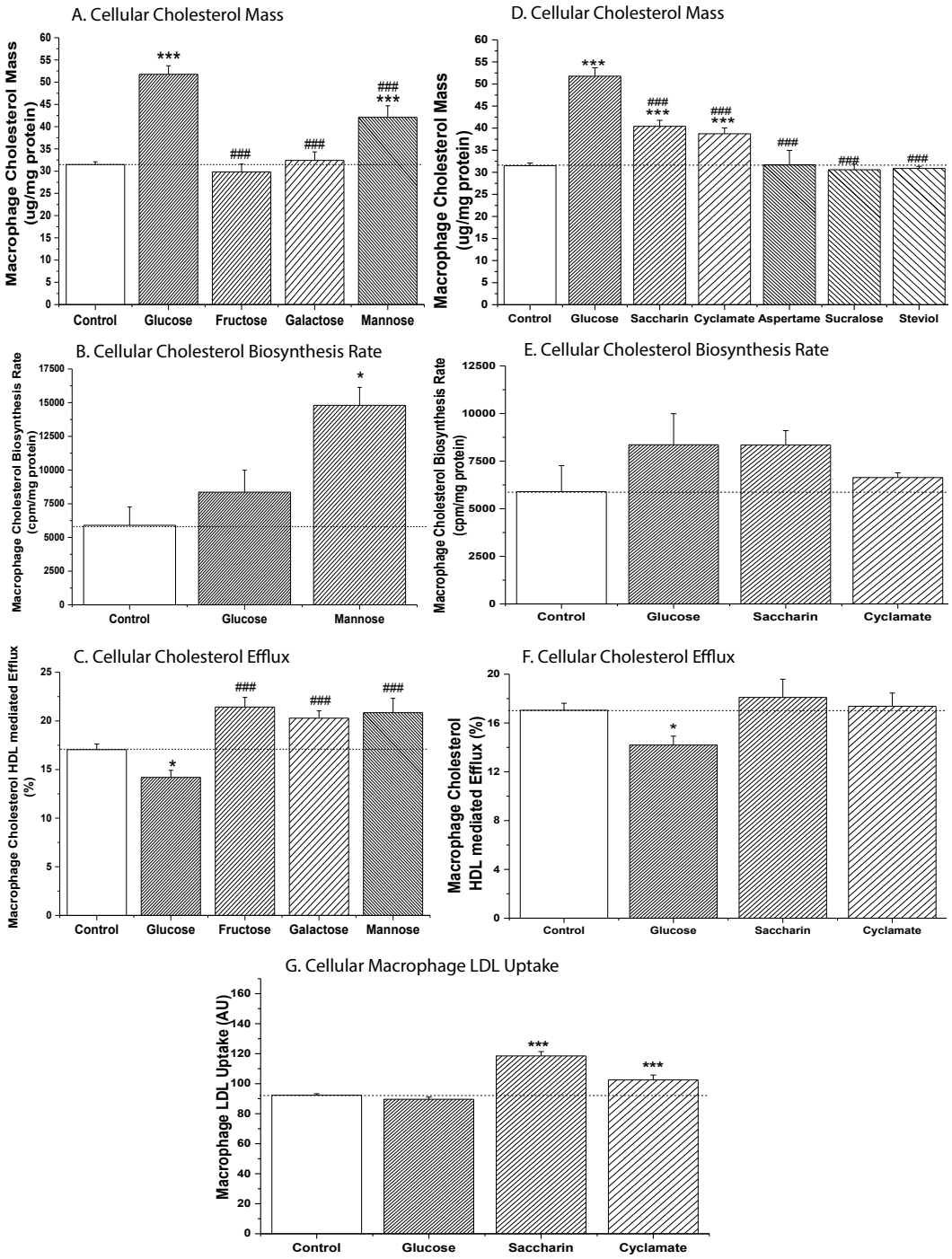

Figure 6: Effects of monosaccharides or artificial sweeteners on J774A.1 macrophage cholesterol metabolism; the effects of each one of the monosaccharides on the macrophage cholesterol metabolism after 18 h of incubation were assessed; (A) Cellular cholesterol mass; (B) Cellular cholesterol biosynthesis rate; and (C) Cellular cholesterol HDL-mediated efflux. The effects of each one of the artificial sweeteners on the macrophage oxidative stress after 18 h of incubation were as well assessed; (D) Cellular cholesterol mass (E) Cellular cholesterol biosynthesis rate; (F) Cellular cholesterol HDL-mediated efflux; and (G) Cellular LDL uptake. (*) P<0.05; (**) P<0.01; (***) P<0.0001 vs. control group and (#) P<0.05, (##) P<0.01, (###) P<0.0001 vs. glucose.

little is known about the potential impact of other sugars or sweeteners [1]. For instance, hyperglycemia is known to enhance atherosclerosis development, and high glucose levels increase macrophage atherogenicity via pro-inflammatory and oxidative stress-related mechanisms [5]. However, the role of monosaccharides other than glucose, as well as that of various disaccharides, or commonly used artificial sweeteners in macrophage foam-cell formation, the key event during early atherogenesis, is currently unknown. The lack of data regarding their possible role in macrophage atherogenicity has led us to compare their effects on the oxidative and lipid status in macrophages.

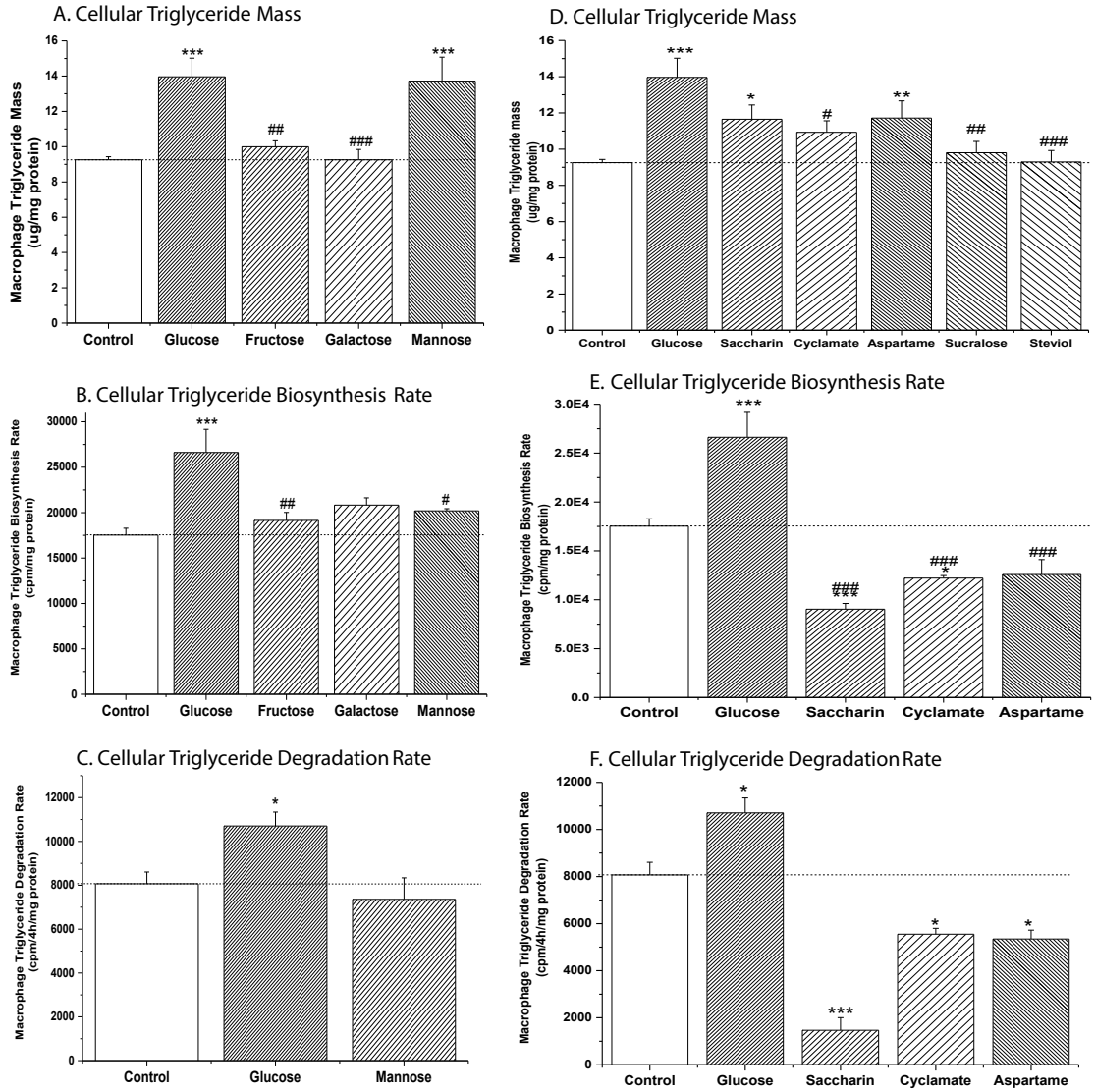

Figure 7: Effects of monosaccharides or artificial sweeteners on J774A.1 macrophage triglyceride metabolism: The effects of each one of the monosaccharides on the macrophage triglyceride metabolism after 18 h of incubation were assessed; (A) Cellular triglyceride mass; (B) Cellular triglyceride biosynthesis rate; (C) Cellular triglyceride degradation rate. The effects of each one of the artificial sweeteners on the macrophage oxidative stress after 18 h of incubation were as well assessed; (D) Cellular triglyceride mass; (E) Cellular triglyceride biosynthesis rate; and (F) Cellular triglyceride degradation rate. (*) P<0.05; (**) P<0.01; (***) P<0.0001 vs. control group; (#) P<0.05; (##) P<0.01; (###) P<0.0001 vs. glucose.

Overall, the findings of the current study demonstrate that at non-cytotoxic concentrations, glucose, mannose, lactose and cyclamate significantly affect macrophage oxidative status and cellular lipid accumulation with the most prominent effects observed for glucose-treated cells. These findings suggest that glucose has the most potent pro-atherogenic effects on macrophage foam cell formation. Evidence was gathered to show that sucrose consumption is a risk factor for coronary heart diseases [48,49]. In addition, it was reported that blood glucose level is a better predictor of atherosclerosis development than serum cholesterol, and that sucrose can increase serum cholesterol level, and aggravates carbohydrate-induced hypertriglyceridemia [48].

The current investigation revealed that glucose was the most potent pro-oxidative/pro-atherogenic in the macrophage model system. Glucose significantly increased the oxidative parameters in macrophages including ROS generation and protein oxidation, while decreasing the cellular antioxidants thiol and glutathione contents, as well as the cellular anti-oxidant PON2. Glucose also caused a significant cholesterol accumulation in J774A.1 cultured macrophages, mainly through decreasing HDL-mediated cholesterol efflux. Moreover, glucose increased the macrophage triglyceride mass via enhancement of their cellular biosynthesis rate. These findings are in line with previous reports on the pro-oxidative impact of glucose [50-53]. Under high glucose levels, more glucose flux through the glycolytic pathway, to produce pyruvate and acetyl-CoA, thus leading to enhanced NADH production, and electron pressure on the mitochondrial electron transport chain [51,52]. Such an electron pressure places a heavy burden on mitochondrial complex I to oxidize more NADH to NAD^+, in an attempt to improve the pseudo-hypoxic condition, leading to increased electron leakage to produce superoxide and oxidative stress [50-52].

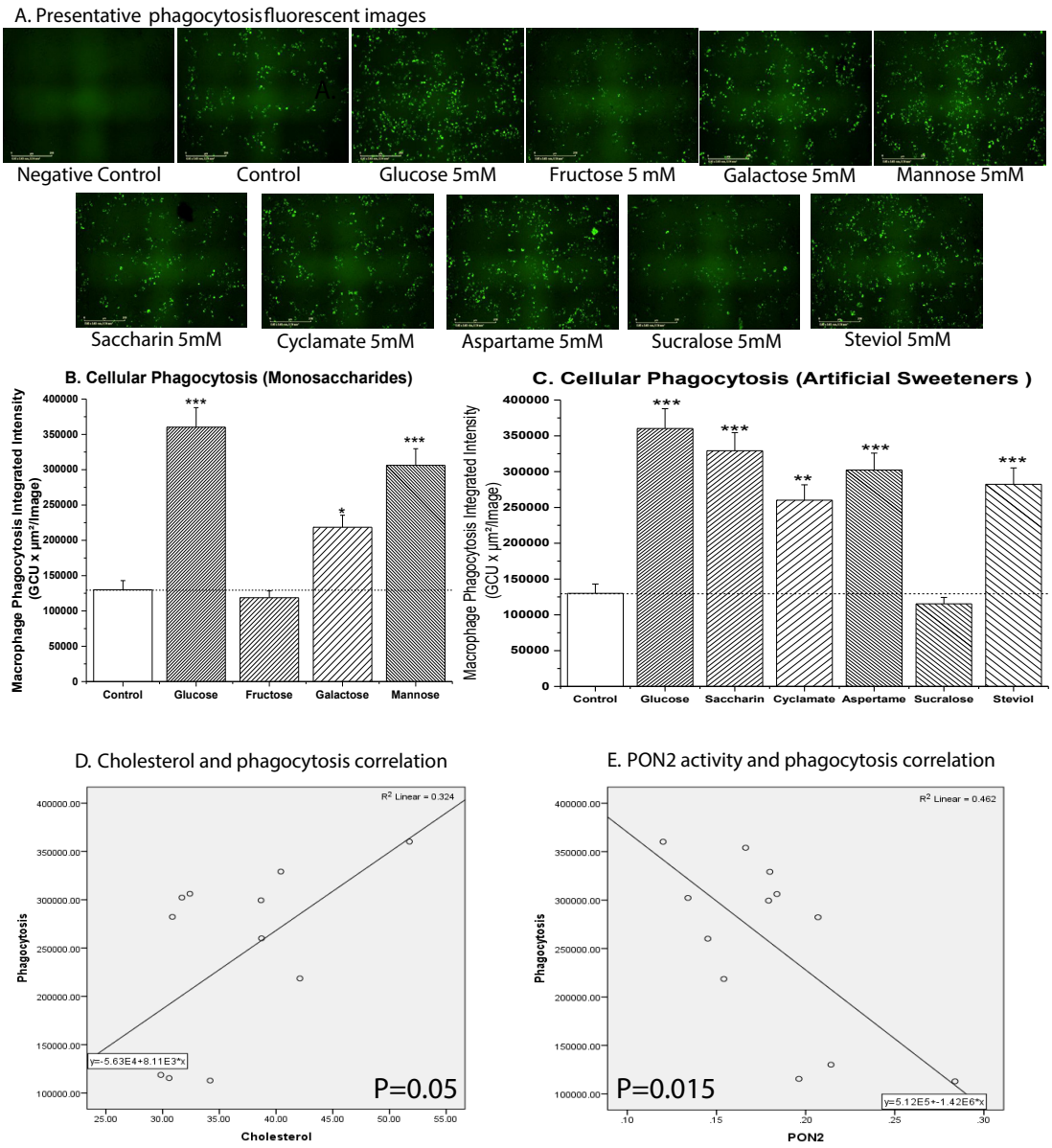

Figure 8: Effects of monosaccharides or artificial sweeteners on J774A.1 macrophage zymosan bioparticles phagocytosis using IncuCyte Phagocytosis assay: The effects of each one of the monosaccharides or artificial sweeteners on the macrophage phagocytosis capacity after 18 h of incubation were assessed. (A) Presentative phagocytosis fluorescent images; (B) Phagocytosis level of monosaccharides; (C) Phagocytosis level of artificial sweeteners; (D) Correlation chart between phagocytosis level and cellular cholesterol content; and (E) Correlation chart between phagocytosis level and cellular PON2 lactonase activity level, following 18 h of incubation. (*) P<0.05; (**) P<0.01; (***) P<0.0001 vs. control group.

In the present study, we show that MPM from treated mice with the monosaccharides mannose and fructose, as well as the disaccharides lactose and sucrose, significantly increased macrophage ROS generation, and showed a trend of increment in both cholesterol and triglyceride mass. However, no significant changes in protein expression or mRNA in the key regulators of the cholesterol and triglyceride metabolism systems, nor on antioxidant regulators, were observed. Compared to glucose, little is known about the impact of the other monosaccharides and disaccharides on macrophage oxidative status, lipid accumulation, and foam cell formation. Previous studies in serum showed that chronic feeding with fructose or sucrose leads to hyperinsulinemia, high triglyceride levels, as well as a significant loss of insulin sensitivity in rodent models [54-57]. Another study showed that high fructose diet in rats, was associated with an increase in ROS production and other oxidative stress markers in the aorta and the heart; and also. cardiac hypertrophy, and an increase in blood pressure [58].

The pro-atherogenic properties of lactose observed in the current study include increased cellular oxidative stress, increased cholesterol content, and to a lesser extent triglyceride content, are in line with previous studies conducted on rhesus monkeys that were fed an atherogenic diet with sucrose or lactose [59]. Treatment of rhesus monkeys with sucrose or lactose enriched diet resulted in more proliferative areas in the intimal lesions, together with hypercholesterolemia (lactose>sucrose), and showed a tendency

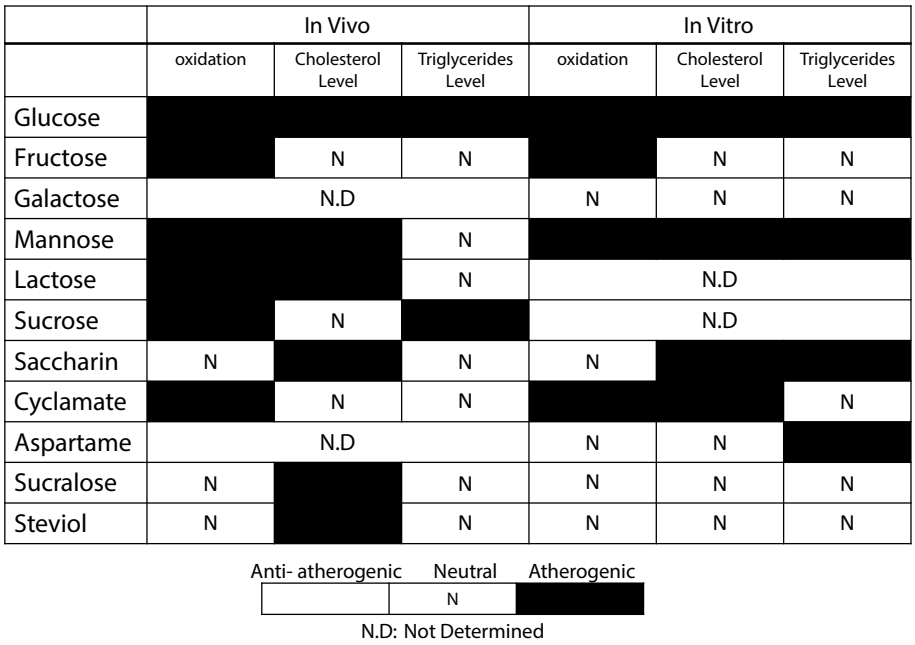

Figure 9: The Impact of monosaccharides, disaccharides and artificial sweeteners on atherogenisity; the effects of each one of the monosaccharides, disaccharides or artificial sweeteners on the macrophage atherogenisity were summarized.

towards increased aortic cholesterol content when compared to control group [59]. Studies showed a positive correlation between carbohydrates and triglycerides levels in serum from humans or rodent models [60-64]. Increasing sucrose proportions in a controlled diet (44% of energy as carbohydrate) resulted in elevations of fasting plasma triacylglycenol concentrations in normal men [65]. Nonetheless, as previously mentioned, there is no data on how different sugars affect lipogenesis, triglyceride clearance or VLDL-particle production rate [60].

The association between artificial sweeteners and atherosclerosis development was poorly studied till now. Few studies reported that daily consumption of diet soft drinks, containing artificial sweeteners, are associated with increased risk for cardiovascular events [26], coronary heart disease in women [24], metabolic syndrome, and type 2 diabetes [66]. Yet, no mechanism to support these correlations was suggested. We observed that all sweeteners have a trend of increment in MPM oxidative stress. The artificial sweeteners, except for cyclamate, showed significant increment in MPM cholesterol content but not triglyceride content, probably as a result of a combination of an increase in cholesterol biosynthesis, reduction in HDL-mediated efflux and increment in lipoprotein uptake. In the *in-Vitro* study, aspartame increased J774A.1 macrophage oxidative stress and triglyceride mass, whereas cyclamate increased macrophage oxidative stress and cholesterol mass, and saccharin increased cholesterol mass and triglyceride mass but not oxidative stress.

It was previously shown that high concentrations of aspartame and saccharin (25-100 mM) impaired the beneficial anti–atherogenic activities of HDL, induced embryonic toxicity and increased ROS production [30].

Another study showed that treatment of apolipoproteinA-1 and HDL with 3mM of saccharin or aspartame for 16h resulted in the loss of their antioxidant and phospholipid binding activities, and accelerated the senescence of human dermal fibroblasts [29]. High artificial sweeteners intake was shown to be associated with increased BMI and body fat percentage in males and females [22,67]. Some studies attributed the effects of artificial sweeteners on lipid levels to the G protein-coupled sweet taste receptors T1R2 and T1R3 which suppressed both basal and stimulated lipolysis [68,69].

Phagocytosis is a key process in atherogenesis, with the ability to increase plaque progression and instability [70, 71]. We showed now that glucose increased the phagocytosis capability of macrophages by almost two-folds. This finding is supported by previous studies showing that phagocytosis by murine macrophages is a glucose-dependent process which is positively correlated with glucose and mannose transport through the GLUT1 transporter [72,73]. The effects of the artificial sweeteners on macrophage phagocytosis capability were investigated now, for the first time, and demonstrated that saccharin, cyclamate, and aspartame increased the macrophage phagocytosis capability in correlation with increased macrophage cholesterol acclamation. These findings suggest that the above artificial sweeteners may affect the macrophage phagocytosis capability via modulation of their cholesterol content. Indeed, the correlation between cholesterol levels and phagocytosis was previously reported [74-78]. Moreover, cellular cholesterol has been reported to influence phagocytosis through regulation of the macrophage membrane fluidity [79].

Conclusion

In conclusion, glucose have the most potent pro-oxidative and pro-atherogenic effects among other sugars and sweeteners, making it the less recommended sweetener, as evident from its ability to induce ROS generation, protein oxidation, depletion of antioxidants, increment in cellular cholesterol and triglycerides mass, and augmentation of macrophage phagocytosis capability. Moreover, in cultured macrophages, as well as in mice macrophages, fructose, mannose, lactose, saccharin, cyclamate and aspartame, all, increase macrophage oxidative status, and lipids accumulation, thus leading to macrophage foam cell formation.

References

1. Frostegard J (2005) SLE, atherosclerosis and cardiovascular disease. J Intern Med 257:485- 95.
2. Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, et al. (2016) Heart disease and stroke statistics-2016 update: A report from the american heart association. Circulation 133: e38-e360.
3. Dickhout JG, Basseri S, Austin RC (2008) Macrophage function and its impact on atherosclerotic lesion composition, progression, and stability: the good, the bad, and the ugly. Arterioscler Thromb Vasc Biol 28: 1413-1415.
4. Rom O, Aviram M (2016) Endogenous or exogenous antioxidants vs. pro-oxidants in macrophage atherogenicity. Curr Opin Lipidol 27: 204-206.
5. Fitch C, Keim KS, (2012) Position of the academy of nutrition and dietetics: use of nutritive and nonnutritive sweeteners. J Acad Nutr Diet 112: 739-758.
6. Kaplan M, Aviram M, Hayek T (2012) Oxidative stress and macrophage foam cell formation during diabetes mellitus-induced atherogenesis: Role of insulin therapy. Pharmacol Ther 136:175-185.
7. Hayek T, Hussein K, Aviram M, Coleman R, Keidar S, et al. (2005) Macrophage foam-cell formation in streptozotocin-induced diabetic mice: Stimulatory effect of glucose. Atheroscler 183: 25-33.
8. Hayek T, Kaplan M, Kerry R, Aviram M (2007) Macrophage NADPH oxidase activation, impaired cholesterol fluxes and increased cholesterol biosynthesis in diabetic mice: A stimulatory role for D-glucose. Atheroscler 195: 277-286.
9. Fukuhara-Takaki K, Sakai M, Sakamoto Y, Takeya M, Horiuchi S (2005) Expression of class A scavenger receptor is enhanced by high glucose in vitro and under diabetic conditions *in vivo*: One mechanism for an increased rate of atherosclerosis in diabetes. J Biol Chem 280: 3355-3364.
10. Gantman A, Fuhrman B, Aviram M, Hayek T(2010) High glucose stimulates macrophage SR-BI expression and induces a switch in its activity from cholesterol efflux to cholesterol influx. Biochem Biophys Res Commun 391: 523-528.
11. Griffin E, Re A, Hamel N, Fu C, Bush H, et al. (2001) A link between diabetes and atherosclerosis: Glucose regulates expression of CD36 at the level of translation. Nat Med7: 840-846.
12. Kaplan M, Kerry R, Aviram M, Hayek T(2008) High glucose concentration increases macrophage cholesterol biosynthesis in diabetes through activation of the sterol regulatory element binding protein 1 (SREBP1): inhibitory effect of insulin. J Cardiovasc Pharmacol 52: 324-332.
13. Mauerer R, Ebert S, Langmann T (2009) High glucose, unsaturated and saturated fatty acids differentially regulate expression of ATP-binding cassette transporters ABCA1 and ABCG1 in human macrophages. Exp Mol Med 41: 126-132.
14. Meilin E, Aviram M, Hayek T (2010) Paraoxonase 2 (PON2) decreases high glucose-induced macrophage triglycerides (TG) accumulation, *via* inhibition of NADPH-oxidase and DGAT1 activity: studies in PON2- deficient mice. Atheroscler 208: 390-395.
15. Chung J, An SH, Kang SW, Kwon K (2016) Ursodeoxycholic acid (UDCA) exerts anti-atherogenic effects by inhibiting rage signaling in diabetic atherosclerosis. PloS one 11: e0147839.
16. Fu Y, Maianu L, Melbert BR, Garvey WT (2004) Facilitative glucose transporter gene expression in human lymphocytes, monocytes, and macrophages: a role for GLUT isoforms 1, 3 and 5 in the immune response and foam cell formation. Blood Cells Mol Dis 32: 182-190.
17. Cura AJ, Carruthers A (2012) Role of monosaccharide transport proteins in carbohydrate assimilation, distribution, metabolism, and homeostasis. Compr Physiol
18. Everett KD, Barghouthi S, Speert DP (1996) *In vitro* culture of murine peritoneal and alveolar macrophages modulates phagocytosis of Pseudomonas aeruginosa and glucose transport. J Leukoc Biol 59: 539-544.
19. Collins AR, Meehan WP, Kintscher U, Jackson S, Wakino S, et al. (2001) Troglitazone inhibits formation of early atherosclerotic lesions in diabetic and nondiabetic low density lipoprotein receptor-deficient mice. Arterioscler Thromb Vasc Biol 21: 365-371.
20. Suzuki M, Yamamoto D, Suzuki T, Fujii M, Suzuki N, et al. (2006) High fat and high fructose diet induced intracranial atherosclerosis and enhanced vasoconstrictor responses in non-human primate. Life Sci 80: 200-204.
21. Cohn ZA, Ehrenreich BA (1969) The uptake, storage, and intracellular hydrolysis of carbohydrates by macrophages. J Exp Med 129: 201-225.
22. Swithers SE (2013) Artificial sweeteners produce the counterintuitive effect of inducing metabolic derangements. Trends Endocrinol Metab 24: 431-441.
23. Sylvetsky AC, Welsh JA, Brown RJ, Vos MB (2012) Low-calorie sweetener consumption is increasing in the United States. Am J Clin Nutr 96: 640-646.
24. Fung TT, Malik V, Rexrode KM, Manson JE, Willett WC, et al. (2009) Sweetened beverage consumption and risk of coronary heart disease in women. Am J Clin Nutr 89: 1037-1042.
25. De Koning L, Malik VS, Kellogg MD, Rimm EB, Willett WC, et al. (2012) Sweetened beverage consumption, incident coronary heart disease and biomarkers of risk in men. Circ 125: 1735-1741.
26. Gardener H, Rundek T, Markert M, Wright CB, Elkind MS, et al. (2012) Diet soft drink consumption is associated with an increased risk of vascular events in the Northern Manhattan Study. J Gen Intern Med 27: 1120-1126.
27. Otero-Losada ME, Mc Loughlin S, Rodriguez-Granillo G, Muller A, Ottaviano G, et al. (2013) Metabolic disturbances and worsening of atherosclerotic lesions in ApoE-/- mice after cola beverages drinking. Cardiovasc Diabetol 12: 57.
28. Otero-Losada M, Cao G, Mc Loughlin S, Rodriguez-Granillo G, Ottaviano G, et al. (2014) Rate of atherosclerosis progression in ApoE-/- mice long after discontinuation of cola beverage drinking. PloS one 9: e89838.
29. Jang W, Jeoung NH, Cho KH (2011) Modified apolipoprotein (apo) A-I by artificial sweetener causes severe premature cellular senescence and atherosclerosis with impairment of functional and structural properties of apoA-I in lipid-free and lipid-bound state. Mol Cells 31: 461-470.
30. Kim JY, Park KH, Kim J, Choi I, Cho KH (2015) Modified high-density lipoproteins by artificial sweetener, aspartame, and saccharin, showed loss of anti-atherosclerotic activity and toxicity in zebrafish. Cardiovasc Toxicol 15: 79-89.
31. Geeraert B, Crombe F, Hulsmans M, Benhabiles N, Geuns JM, et al. (2010) Stevioside inhibits atherosclerosis by improving insulin signaling and antioxidant defense in obese insulin-resistant mice. Int J Obes (Lond) 34:569-577.
32. Paigen B, Holmes PA, Mitchell D, Albee D (1987) Comparison of atherosclerotic lesions and HDL-lipid levels in male, female, and testosterone-treated female mice from strains C57BL/6, BALB/c, and C3H. Atherosclerosis 64:215-21.
33. Whitman SC (2004) A practical approach to using mice in atherosclerosis research. The Clinical Biochemist Reviews 25:81-93.
34. FDA. Food Additives & Ingredients-High-Intensity Sweeteners. 2016.
35. El-Saadani M, Esterbauer H, El-Sayed M, Goher M, Nassar AY, et al. (1989) A spectrophotometric assay for lipid peroxides in serum lipoproteins using a commercially available reagent. J Lipid Res 30: 627-630.
36. Buege JA, Aust SD (1978) Microsomal lipid peroxidation. Methods Enzymol 52: 302-310.
37. Rom O, Korach-Rechtman H, Hayek T, Danin-Poleg Y, Bar H, et al. (2016) Acrolein increases macrophage atherogenicity in association with gut microbiota remodeling in atherosclerotic mice: protective role for the polyphenol-rich pomegranate juice. Arch Toxicol 91: 1709-1725.
38. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193: 265-275.
39. Rom O, Volkova N, Nandi S, Jelinek R, Aviram M, et al. (2016) Pomegranate juice polyphenols induce macrophage death *via* apoptosis as opposed to necrosis induced by free radical generation: A central role for oxidative stress. J Cardiovasc Pharmacol 68: 106-114.
40. Van der Windt GJ, Chang CH, Pearce EL (2016) Measuring bioenergetics in T cells using a seahorse extracellular flux analyzer. Curr Protoc Immunol 3: 16B,1-3; 16B,14.
41. Abu-Saleh N, Aviram M, Hayek T (2016) Aqueous or lipid components of atherosclerotic lesion increase macrophage oxidation and lipid accumulation. Life Sci 154: 1-14.
42. Selmeci L, Seres L, Antal M, Lukacs J, Regoly-Merei A, et al. (2005) Advanced oxidation protein products (AOPP) for monitoring oxidative stress in critically ill patients: a simple, fast and inexpensive automated technique. Clin Chem Lab Med 43: 294-297.
43. Gaidukov L, Tawfik DS (2005) High affinity, stability, and lactonase activity of serum paraoxonase PON1 anchored on HDL with ApoA-I. Biochemistry 44: 11843-11854.

44. Hu ML, Dillard CJ, Tappel AL (1988) *In vivo* effects of aurothioglucose and sodium thioglucose on rat tissue sulfhydryl levels and plasma sulfhydryl reactivity. Agents and Actions 25: 132-138.

45. Aviram M (1983) Plasma lipoprotein separation by discontinuous density gradient ultracentrifugation in hyperlipoproteinemic patients. Biochemical Medicine. 30: 111-118.

46. Smythe CD, Skinner VO, Bruckdorfer KR, Haskard DO, Landis RC, et al. (2003) The state of macrophage differentiation determines the TNF alpha response to nitrated lipoprotein uptake. Atherosclerosis 170: 213-221.

47. Etxebarria A, Benito-Vicente A, Stef M, Ostolaza H, Palacios L, et al. (2015) Activity-associated effect of LDL receptor missense variants located in the cysteine-rich repeats. Atherosclerosis 241: e119.

48. Kearns CE, Schmidt LA, Glantz SA (2016) Sugar industry and coronary heart disease research: A historical analysis of internal industry documents. JAMA Intern Med 176: 1680-1685.

49. McGandy RB, Hegsted DM, Stare FJ (1967) Dietary fats, carbohydrates and atherosclerotic vascular disease. N Engl J Med 277: 242-247.

50. Rebolledo OR, Actis Dato SM (2005) Postprandial hyperglycemia and hyperlipidemia-generated glycoxidative stress: Its contribution to the pathogenesis of diabetes complications. Eur Rev Med Pharmacol Sci 9: 191-208.

51. Behl T, Kaur I, Kotwani A (2016) Implication of oxidative stress in progression of diabetic retinopathy. Survey of Ophthalmology 61: 187-196.

52. Kim JA, Wei Y, Sowers JR (2008) Role of mitochondrial dysfunction in insulin resistance. Circ Res. 102:401-414.

53. Hirst J, King MS, Pryde KR (2008) The production of reactive oxygen species by complex I. Biochemical Society transactions 36: 976-980.

54. Tobey TA, Mondon CE, Zavaroni I, Reaven GM (1982) Mechanism of insulin resistance in fructose-fed rats. Metabolism: Clinical and Experimental 31: 608-612.

55. Wright DW, Hansen RI, Mondon CE, Reaven GM (1983) Sucrose-induced insulin resistance in the rat: Modulation by exercise and diet. The American Journal of Clinical Nutrition 38: 879-83.

56. Cohen AM, Teitelbaum A (1964) Effect of dietary sucrose and starch on oral glucose tolerance and insulin- like activity. The American Journal of Physiology 206: 105-108.

57. Vrana A, Fabry P (1983) Metabolic effects of high sucrose or fructose intake. World review of nutrition and dietetics 42: 56-101.

58. Delbosc S, Paizanis E, Magous R, Araiz C, Dimo T, et al. (2005) Involvement of oxidative stress and NADPH oxidase activation in the development of cardiovascular complications in a model of insulin resistance, the fructose-fed rat. Atherosclerosis. 179: 43-49.

59. Armstrong ML, Megan MB, Cheng FH, Warner ED (1976) Dietary disaccharides in experimental atherosclerosis in rhesus monkeys. Experimental and Molecular Pathology 24: 302-319.

60. Parks EJ (2001) Effect of dietary carbohydrate on triglyceride metabolism in humans. The Journal of Nutrition131: 2772S-2774S.

61. Parks EJ, Hellerstein MK (2000) Carbohydrate-induced hypertriacylglycerolemia: Historical perspective and review of biological mechanisms. The American Journal of Clinical Nutrition 71: 412-433.

62. Frayn KN, Kingman SM (1995) Dietary sugars and lipid metabolism in humans. The American Journal of Clinical Nutrition 62: 250S-61S.

63. Xue CY, Kageyama H, Kashiba M, Kobayashi A, Osaka T, et al. (2001) Different origin of hypertriglyceridemia induced by a high-fat and a high-sucrose diet in ventromedial hypothalamic-lesioned obese and normal rats. International Journal Of Obesity And Related Metabolic Disorders: Journal of the International Association for the Study of Obesity 25: 434-438.

64. Howard BV, Wylie-Rosett J (2002) Sugar and cardiovascular disease: A statement for healthcare professionals from the Committee on Nutrition of the Council on Nutrition, Physical Activity, and Metabolism of the American Heart Association. Circulation106: 523-527.

65. Reiser S, Bickard MC, Hallfrisch J, Michaelis OEt, Prather ES (1981) Blood lipids and their distribution in lipoproteins in hyperinsulinemic subjects fed three different levels of sucrose. The Journal of Nutrition 111: 1045-57.

66. Nettleton JA, Lutsey PL, Wang Y, Lima JA, Michos ED, et al. (2009) Diet soda intake and risk of incident metabolic syndrome and type 2 diabetes in the Multi-Ethnic Study of Atherosclerosis (MESA). Diabetes Care 32: 688-694.

67. Laska MN, Murray DM, Lytle LA, Harnack LJ (2012) Longitudinal associations between key dietary behaviors and weight gain over time: Transitions through the adolescent years. Obesity 20: 118-125.

68. Nelson G, Hoon MA, Chandrashekar J, Zhang Y, Ryba N J, et al. (2001) Mammalian sweet taste receptors. Cell 106, 381-390.

69. Simon BR1, Parlee SD, Learman BS, Mori H, Scheller EL, et al. (2013) Artificial sweeteners stimulate adipogenesis and suppress lipolysis independently of sweet taste receptors. J Biol Chem 8: 288.

70. Schrijvers DM, De Meyer GR, Herman AG, Martinet W (2007) Phagocytosis in atherosclerosis: Molecular mechanisms and implications for plaque progression and stability. Cardiovascular Research73: 470-480.

71. Aderem A, Underhill DM (1999) Mechanisms of phagocytosis in macrophages. Annu Rev Immunol 17: 593-623.

72. Barghouthi S, Everett KD, Speert DP (1995) Nonopsonic phagocytosis of Pseudomonas aeruginosa requires facilitated transport of D-glucose by macrophages. Journal of Immunology. 154:3420-3428.

73. Wong SY, Guerdoud LM, Cantin A, Speert DP (1999) Glucose stimulates phagocytosis of unopsonized Pseudomonas aeruginosa by cultivated human alveolar macrophages. Infect Immun 67: 16-21.

74. Nuzum KDF (1914) The Effect of Cholesterol on Phagocytosis. The Journal of Infectious Diseases. 15: 472-482.

75. Tunnicliff R (1923) The Influence of Cholesterol on Phagocytosis. The Journal of Infectious Diseases. 33: 285-288.

76. Amiel E, Nicholson-Dykstra S, Walters JJ, Higgs H, Berwin B (2007) Scavenger receptor-A functions in phagocytosis of E. coli by bone marrow dendritic cells. Experimental Cell Research 313: 1438-1448.

77. Gatfield J, Pieters J (2000) Essential role for cholesterol in entry of mycobacteria into macrophages. Science. 288: 1647-1650.

78. Peyron P, Bordier C, N'Diaye EN, Maridonneau-Parini I (2000) Nonopsonic phagocytosis of Mycobacterium kansasii by human neutrophils depends on cholesterol and is mediated by CR3 associated with glycosylphosphatidylinositol-anchored proteins. Journal of Immunology 165: 5186-5191.

79. Kannan S, Audet A, Huang H, Chen LJ, Wu M (2008) Cholesterol-rich membrane rafts and Lyn are involved in phagocytosis during *Pseudomonas aeruginosa* infection. Journal of Immunology 180: 2396-2408