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In vitro bioassay investigations of the endocrine disrupting potential of steviol glycosides and their metabolite steviol, components of the natural sweetener *Stevia*

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Abstract

The food industry is moving towards the use of natural sweeteners such as those produced by *Stevia rebaudiana* due to the number of health and safety concerns surrounding artificial sweeteners. Despite the fact that these sweeteners are natural; they cannot be assumed safe. Steviol glycosides have a steroidal structure and therefore may have the potential to act as an endocrine disruptor in the body. Reporter gene assays (RGAs), H295R steroidogenesis assay and Ca^{2+} fluorimetry based assays using human sperm cells have been used to assess the endocrine disrupting potential of two steviol glycosides: stevioside and rebaudioside A, and their metabolite steviol. A decrease in transcriptional activity of the progestagen receptor was seen following treatment with 25,000 ng/ml steviol in the presence of progesterone (157 ng/ml) resulting in a 31% decrease in progestagen response (p= <0.01). At the level of steroidogenesis, the metabolite steviol (500-25,000 ng/ml) increased progesterone production significantly by 2.3 fold when exposed to 10,000 ng/ml (p= <0.05) and 5 fold when exposed to 25,000 ng/ml (p=<0.001). Additionally, steviol was found to induce an agonistic response on CatSper, a progesterone receptor of sperm, causing a rapid influx of Ca^{2+}. The response was fully inhibited using a specific CatSper inhibitor. These findings highlight the potential for steviol to act as a potential endocrine disruptor.

Key terms: Endocrine disruptors, *in vitro* bioassays, steviol, reproductive disorders, CatSper; *Stevia*

1. Introduction

Steviol glycosides are high intensity sweeteners that can be isolated and purified from the leaves of *Stevia rebaudiana* (Bertoni). These glycosides of the diterpene derivative steviol consist mainly of stevioside and rebaudioside A. These chemicals have sweetening potentials 200-300 times that of sucrose, are thermally stable and account for approximately 5-10% of dry leaf weight (Soejarto, *et al*., 1982).

The increasing prevalence of diabetes and obesity worldwide, in conjunction with a growing concern over the safety of chemical sweeteners such as aspartame has led to a growing demand for natural non-calorie sweeteners such as steviol glycosides which are presumed safe to eat and are of acceptable taste. With increasing health consciousness, the use of steviol glycosides as a food additive is now encouraged to slow down the world-wide sugar consumption and therefore contribute towards combating the increasing diabetes and obesity rates (Brahmachari, *et al*., 2011). Steviol glycosides are now authorised for use in a range of commonly consumed food products (The European Commission, 2011), resulting in steviol equivalents within a range of 20,000 up to 3,300,000 ng/ml abundantly present in foods (Table 1).

Table 1: Examples of authorised uses of steviol glycosides (E-Number 960, expressed as steviol equivalents) in food.

Food category	Maximum level steviol equivalents (ng/ml or ng/g)	Restrictions/Exceptions
Cocoa and Chocolate products; as covered by Directive 2000/36/EC	270,000	Only energy-reduced or with no added sugars
Chewing gum	3,300,000	Only with no added sugar
Flavoured Drinks	80,000	Only energy reduced or with no added sugar
Potato – cereal, flour or starch-based snacks	20,000	
Jam, Jellies and marmalades and sweetened chestnut puree; **as defined by Directive 2001/113/EC**	200,000	Only energy-reduced jams, jellies and marmalades
Fruit and vegetables (**preparations excluding compote**)	200,000	Only energy-reduced

The safety of steviol glycosides for the proposed use as food additives has been evaluated by the Joint Expert Committee on Food Additives (JECFA), a scientific advisory body of Food and Agriculture Organization (FAO) of the United Nations, and the World Health Organisation (WHO). JECFA have established an ADI (Acceptable Daily Intake) for steviol glycosides (expressed as steviol equivalents) of 4 mg/kg bw/day (FAO, 2010). This ADI takes into account a no-observed-adverse-effect-level (NOAEL) and applies a 100-fold safety uncertainty factor extrapolated from a 2-year carcinogenicity study on rats consuming 2.5% stevioside in the diet, equating to 967 mg stevioside/kg bw/day or 388 mg steviol

equivalents/kg bw/day (Xili *et.al.,* 1992). However, conservative estimates of steviol glycoside exposure, both in adults and in children, suggest that the ADI would in some cases be exceeded at the maximum proposed use levels (The European Commission, 2011). Consumption studies demonstrate that intact steviol glycosides are poorly absorbed by humans after oral exposure (Pawar, *et al*., 2013). However, they may be hydrolysed to the metabolite steviol by intestinal microflora in the colon, where the majority is absorbed across the gut wall and the rest is excreted in the faeces. To aid excretion from the body, absorbed steviol is rapidly transformed to steviol glucuronide in the liver via conjugation to an acyl-glucuronide (Brusick, 2008). Consequently, while steviol glycosides and steviol are not detected in the blood or urine of human subjects, steviol glucuronide has been reported in urine and steviol in faeces (Wheeler, et al., 2008).

Human studies into "safe" levels of steviol glycoside consumption have shown that daily doses of rebaudioside A up to 1,000,000 ng/person/day were well-tolerated by individuals with type-2 diabetes mellitus or normal glucose metabolism (Maki *et al.*, 2008). This dose equates to approximately 16,600 ng/kg bw/day for a 60 kg person and corresponds to approximately 330,000 ng steviol equivalents/person/day or to 5,500 ng steviol equivalents/kg bw/day (Maki *et al.*, 2008).

Steviol glycosides and steviol possess a steroidal structure (*Fig.1*) and therefore may have the potential to act as an endocrine disrupting chemical (EDC).

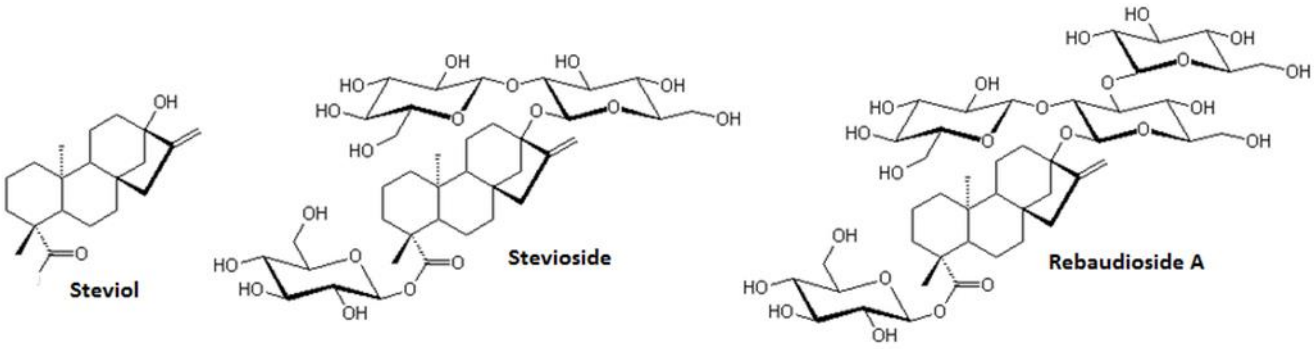


Figure 1: Chemical structure of steviol, stevioside and rebaudioside A

The WHO defines an EDC as "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations" (WHO, 2002). It is now known that EDCs can act via multiple mechanisms within the cell and body. These mechanisms may include the mimicking or blocking of transcriptional activation elicited by naturally present hormones via binding to hormone receptors or interference with hormone production, secretion and control systems in the steroidogenesis pathway (Tabb and Blumberg, 2005).

A limited number of studies have been presented to date on the effects of steviol glycosides on the endocrine system. Some studies conversely report no adverse effects on the reproduction, organs, sperm or foetal development of male or female rats (EFSA, 2010). The majority of these studies have used *Stevia rebaudiana* extracts as opposed to the pure steviol glycosides.

In vitro bioassays are extremely useful tools for the detection and mechanistic study of EDCs (Connolly *et al.,* 2011). The current study aims to investigate the endocrine disrupting activity of the natural sweeteners stevioside and rebaudioside A and their metabolite steviol. Mammalian reporter gene assays (RGAs) incorporating natural steroid receptors have been used to assess effects on nuclear receptor transcriptional activity (Willemsen *et al.,* 2004), while the H295R human adrenal carcinoma cell line which has all the important enzymes and genes needed for steroidogenesis has been used as a model to study effects on hormone production (Hecker and Giesy, 2008). Ca^{2+} fluorimetry based assays using human sperm cells, have also been used to assess the effects of EDCs on the Ca^{2+} channel of sperm (CatSper), which has progesterone as its natural ligand (Schiffer *et. al.,* 2014; Tavares *et.al.,* 2013). CatSper regulates several important sperm functions and is absolutely required for male fertility (Smith *et.al.,* 2013).

To the best of our knowledge this is the first reported study investigating the endocrine disrupting potential of the natural sweeteners stevioside and rebaudioside A and their metabolite steviol at the level of nuclear receptor transcriptional activity using oestrogen, androgen, progestagen and glucocorticoid RGAs, on steroidogenesis using the H295R steroidogenesis model and on the non-genomic progesterone receptor of sperm, CatSper.

2. Methods

2.1 Chemicals and reagents

Cell culture reagents were supplied by Life Technologies (Paisley, UK). The standards 17β-oestradiol, testosterone, hydrocortisone, progesterone, steviol, stevioside, rebaudioside A and forskolin were obtained from Sigma-Aldrich (Poole, Dorset, UK). Dimethyl sulfoxide (DMSO) and thiazolyl blue tetrazolium bromide (MTT) were also supplied by Sigma-Aldrich. Lysis reagents and luciferase assay system was purchased from Promega (Southampton, UK). Fluorescent Ca^{2+} indicator Fluo-4 was provided by Invitrogen (USA). MDL12330A was supplied by Tocris (USA). Human serum albumin was purchased from Irvine Scientific (USA).

2.2 Cell culture

Four reporter gene assay (RGA) cell lines, the MMV-Luc (oestrogen responsive), TARM-Luc (androgen and progestagen responsive), TM-Luc (progestagen responsive) and TGRM-Luc (glucocorticoid and progestagen responsive) were previously developed by transforming human mammary gland cell lines with the luciferase gene under the control of a steroid hormone inducible promoter (Willemsen *et al.*, 2004). The H295R human adrenocortical carcinoma cell line, used in the steroidogenesis model, was obtained from the American Type Culture Collection (ATCC CRL-2128, Manassas, VA, USA).

All cell lines were routinely grown in 75 cm^2 tissue culture flasks (Nunc, Roskilde, Denmark) at 37°C with 5% CO_2 and 95% humidity. The RGA cell lines were cultured in cell culture medium containing Dulbecco's Modified Eagle Medium (DMEM), 10% foetal bovine serum and 1% penicillin streptomycin. For culturing the MMV-Luc cell line, DMEM without phenol red was used due to the weak oestrogenicity of phenol red. The H295R cell line was cultured in cell culture medium containing DMEM with Ham's F-12 nutrient mixture (1:1) supplemented with 1% ITS + Premix and 2.5% NuSerum (BD Biosciences, Bedford, MA, US).

2.3 Cell viability assays

2.3.1 MTT assay

The MTT assay was performed to monitor the toxic effects of test compounds in the RGA cell lines. Briefly, clear flat-bottomed 96-well plates (Nunc, Roskilde, Denmark) were seeded with 4 x 10^5 cells/ml of the appropriate cell line. After 24 h stevioside and steviol (500, 1,000, 5,000, 10,000 and 25,000 ng/ml) and rebaudioside A (5,000, 10,000, 25,000, 50,000 and 100,000 ng/ml) were added to the cells at a final DMSO concentration of 0.1%. Test compounds were then incubated for a further 48 h. Viable cells convert the soluble yellow MTT into insoluble purple formazan by the action of mitochondrial succinate dehydrogenase. Following incubation, supernatant was discarded and 50 µl of MTT solution/well (5mg/ml stock in PBS diluted in 1:2.5 in assay media) was added and cells were incubated for a further 3 h. The supernatant was removed and 200 µl of DMSO was added to each well and incubated for a further 10 min with agitation at 37°C to dissolve the formazan crystals. Optical density was measured using a Sunrise spectrophotometer at 570 nm with a reference filter at 630 nm (TECAN, Switzerland). Samples were analysed in triplicate wells and in three independent experiments. Viability was calculated as a percentage absorbance of the sample when compared with the absorbance of the DMSO solvent control.

2.3.2 AlamarBlue® assay

The viability of the H295R cells was determined using the AlamarBlue® assay. After the removal of the media for hormone analysis, 1 ml of 1:10 (v:v) AlamarBlue® in cell culture medium was added to each well and incubated for 6 h. A 100 µl volume was then removed from each well and added into clear flat-bottomed 96-well microtitre plates (Nunc, Roskilde, Denmark). Using a Sunrise spectrophotometer (TECAN, Switzerland) the absorbance was measured at 570 nm and 600 nm. Viability was calculated as the % absorbance of the sample in comparison with the absorbance of the solvent control (0.1%, v:v DMSO in media).

2.4 Reporter gene assays (RGAs)

The RGA procedure has previously been described by Frizzell *et al.,* (2011). Briefly, cells were seeded at a concentration of 4×10^5 cells/ml, 100 ml/well in specialised white walled, clear and flat bottomed 96-well plates (Greiner Bio-One, Fricken- hausen, Germany). After 24 h, stevioside, steviol (500, 1,000, 5,000, 10,000 and 25,000 ng/ml), rebaudioside A (5,000, 10,000, 25,000, 50,000 and 100,000 ng/ml) and the steroid hormone standards were added to the cells at a final DMSO concentration of 0.1%. The positive controls used with their respective cell lines were: 1.36 ng/ml 17β-oestradiol (MMV-Luc), 14.5 ng/ml testosterone (TARM-Luc), 157 ng/ml progesterone (TM-Luc) and 181 ng/ml hydrocortisone (TGRM-Luc). A solvent control (0.1%, v:v DMSO in media) was also included for each cell line. Antagonist tests were carried out by incubating the test compounds with the relevant positive control for the cell line being tested. The cells were incubated for 48 h. The supernatant was discarded and the cells washed once with PBS prior to lysis with 20 µl cell lysis buffer (Promega, Southampton, UK). Finally, 100 µl luciferase substrate (Promega, Southampton, UK) was injected into each well and luciferase activity measured using a Mithras Multimode Reader (Berthold, Other, Germany). RGAs were performed in triplicate for each

196 experimental point and repeated in at least two independent exposures. The response of the
197 cell lines to the various compounds was measured and compared with the solvent and
198 positive controls.

199 ***2.5 Steroidogenesis assay***

200 As steviol glycosides are metabolised to steviol *in vivo*, steviol was tested at five different
201 concentrations (500, 1,000, 5,000, 10,000 and 25,000 ng/ml). The highest soluble
202 concentration achieved in DMSO was 25,000 ng/ml. The H295R assay was performed as
203 described previously (Gracia *et al.*, 2007). Briefly, the cells were seeded at a concentration of
204 3×10^5 cells/ml, 1 ml per well, in 24-well plates (BD Biosciences, Bedford, MA, US). The
205 cells were allowed to attach for 24 h before removing the media and replacing with fresh
206 media containing the test compounds dissolved in DMSO at a final concentration of 0.1%
207 (v:v). Forskolin was used as a positive control at a concentration of 10uM. A solvent control
208 (0.1%, v:v DMSO in media) was also included. Subsequently, the media was collected from
209 the wells following 48 h incubation and stored at −20°C until hormone quantification was
210 carried out. A 48 hour incubation time allows the concentrations of these hormones to reach a
211 plateau-phase under these conditions. The AlamarBlue® cell viability assay was carried out
212 on the remaining cells in each well. Each experimental point was performed in triplicate in at
213 least two independent exposures.

214 ***2.6 Hormone detection and quantification***

215 Frozen media was thawed prior to analysis. Oestradiol, testosterone and progesterone levels
216 in the media were quantified by enzyme-linked immunosorbent assays (ELISAs)
217 (Immunodiagnostics, Marburg, Germany). These highly specific kits are based on the
218 principle of competitive binding and are intended for the quantitative *in vitro* diagnostic
219 measurement of oestradiol (0–2000 pg/ml), testosterone (0–16 ng/ml) and progesterone (0–40

ng/ml) in serum and plasma, with sensitivities of 9.714 pg/ml, 0.083 ng/ml and 0.045 ng/ml respectively. ELISA kits were carried out according to manufacturer's instructions with the exception of the standard curves which were prepared in the same culture medium used for the H295R assay. Prior to media analysis, it was confirmed that steviol (500-25,000 ng/ml) did not cross-react with the progesterone ELISA antibody (data not included). The intra-assay coefficient of variation was less than 10%. Standard curves were included on each ELISA plate. The mean absorbance obtained from each standard was plotted against its concentration using dose–response curves generated with Graph Pad Prism software.

2.7 Sperm preparation and measurement of changes in intracellular Ca^{2+} concentration

Samples of human semen were obtained from healthy volunteers with their prior consent. Sperm were prepared as described (Schiffer *et.al.*, 2014). Briefly, sperm were purified by a "swim-up" procedure in human tubular fluid (HTF^+) containing (in mM): 97.8 NaCl, 4.69 KCl, 0.2 $MgSO_4$, 0.37 KH_2PO_4, 2.04 $CaCl_2$, 0.33 Na-pyruvate, 21.4 lactic acid, 2.78 glucose, 21 HEPES, and 4 $NaHCO_3$ adjusted between pH 7.3-7.4 with NaOH. After washing, human serum albumin (3 mg/ml) was added to HTF^+. Sperm were incubated for at least 1 h in HTF^+ with 3 mg/ml serum albumin at 37 °C in a 10% CO_2 atmosphere.

Changes in intracellular Ca^{2+} concentration in human sperm were measured in 384 multi-well plates in a fluorescence plate reader (Fluostar Omega, BMG Labtech, Germany) at 30 °C. Sperm were loaded with the fluorescent Ca^{2+} indicator Fluo-4 (10 μM) for 45 min at 37 °C. After incubation, excess dye was removed by centrifugation (700 x g, 10 min, RT). The sperm pellet was resuspended in HTF^+ to 5×10^6 sperm/ml. Aliquots of 50 μl were loaded to the wells of the multi-well plate. Fluorescence was excited at 480 nm and emission was recorded at 520 nm with bottom optics. Fluorescence was recorded before and after injection

of 25 µl (1:3 dilution) of negative control (buffer), positive control (progesterone, 5 µM) and steviol manually with an electronic multichannel pipette. Steviol was dissolved in DMSO to 10 mM and further diluted in HTF^+. Changes in Fluo-4 fluorescence are shown as $\Delta F/F_0$ (%), indicating the percentage change in fluorescence (ΔF) with respect to the mean basal fluorescence (F_0) before addition of steviol, positive control and negative control. For the inhibition experiment, sperm were incubated with MDL12330A for 5 min prior to addition of steviol.

2.8 Statistical Analysis

All values shown are expressed as mean ± standard deviation (SD) of at least two independent exposures for the compounds tested (for two independent exposures n=2 and for three independent exposures n=3). Data from the cell viability, reporter gene, steroidogenesis and Ca^{2+} fluorimetry assays were analysed using Microsoft Excel and Graphpad PRISM 5 software (San Diego, CA). A one way analysis of variance (ANOVA) and Dunnett's Multiple Comparison Test was used to determine significant differences between treatments and the corresponding controls. The mean concentrations were tested for significant difference at the 95% confidence level, a *P*-value of <0.05 was considered as significant ($P \leq 0.05$ *, $P \leq 0.01$ ** and $P \leq 0.001$ ***).

3 Results

3.1 Cell viability and cytotoxicity

*S*tevioside (500-25,000 ng/ml), steviol (500-25,000 ng/ml) and rebaudioside A (5000-100,000 ng/ml) were assessed for cytotoxicity by the MTT assay in the MMV-Luc (oestrogen responsive), TM-Luc (progestagen responsive), TARM-Luc (androgen and progestagen responsive) and TGRM-Luc (glucocorticoid and progestagen responsive) cell lines (*Fig.2*).

Cytotoxicity was observed at the higher concentrations of stevioside in the TARM-Luc (25,000 ng/ml $p \leq 0.001$), TM-Luc (5,000, 10,000, and 25,000 ng/ml, $P \leq 0.001$) and TGRM-Luc (10,000 and 25,000 ng/ml, $P \leq 0.001$) RGA cell lines.

Steviol (500-25,000 ng/ml) was assessed for cytotoxicity in the H295R cell line by the AlamarBlue® assay. Cytotoxicity was not observed at any of the concentrations tested (data not shown). The viability of the cells at the sample concentrations did not differ significantly from the DMSO solvent control ($P \geq 0.05$).

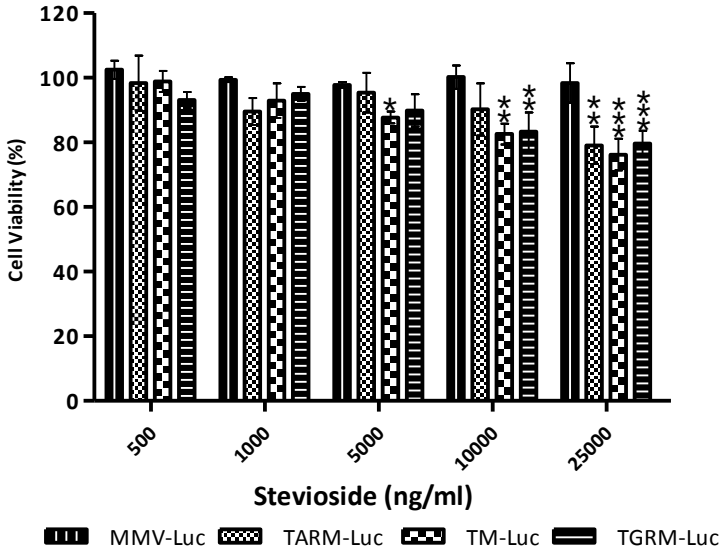


Figure 2: Viability of the RGA cell lines following exposure to (a) 500-25,000 ng/ml stevioside for 48 h compared to the solvent control, as determined in the MTT assay. The MMV-Luc cell line is specific for the detection of oestrogens, TARM-Luc for androgens and progestagens, TM-Luc for progestagens and TGRM-Luc for glucocorticoids and progestagens. Values are means ± SD for three independent exposures in triplicate (n = 3). P = <0.05 (*) P≤ 0.01 (**) P = <0.001 (***)

3.2 Reporter gene assay

An agonist response was not observed for stevioside, steviol or rebaudioside A in the MMV-Luc, TM-Luc, TARM-Luc or TGRM-Luc cell lines (data not shown). Additionally, no antagonist response was seen for stevioside and rebaudioside A in the MMV-Luc, TM-Luc, TARM-Luc or TGRM-Luc cell lines (data not shown). Steviol appeared to exhibit an antagonist response in the progesterone responsive TM-Luc cell line, with a 28.1% reduction in progesterone response at the highest concentration of 25,000 ng/ml ($P \leq 0.05$) (*Fig.3*). At this concentration of steviol, no reduction in cell viability was observed in the MTT assay. Although there was a trend in reduction of glucocorticoid transcriptional activity at 25,000 ng/ml steviol, this reduction was not significant ($P \geq 0.05$).

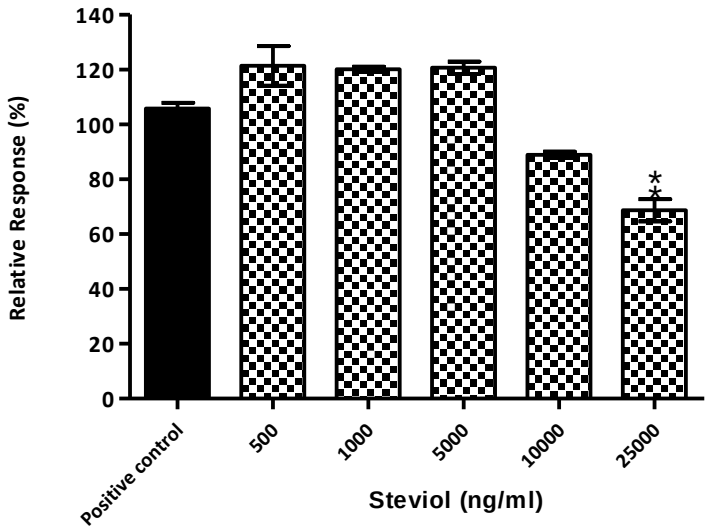


Figure 3: Antagonist effects in the TM-Luc cell line cell lines for 500-25,000 ng/ml steviol. Responses measured are compared to the relative positive control (157 ng/ml progesterone). Response is expressed as the percentage response ± SD for three independent exposures in triplicate (n = 3). P = ≤0.01 (**)

3.3 Hormone production results

Steviol did not induce significant changes in the production of oestradiol and testosterone (data not shown). However, progesterone production increased significantly by 2.3 fold when exposed to 10,000 ng/ml (P = <0.05) and 5 fold when exposed to 25,000 ng/ml (P =<0.001) (*Fig.4*).

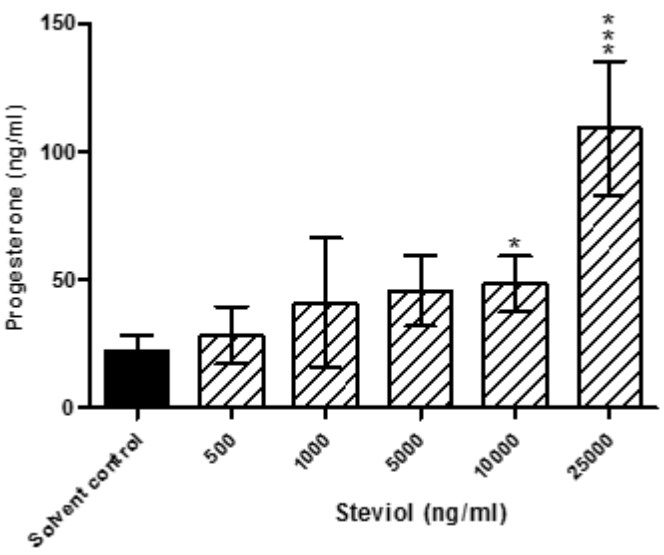
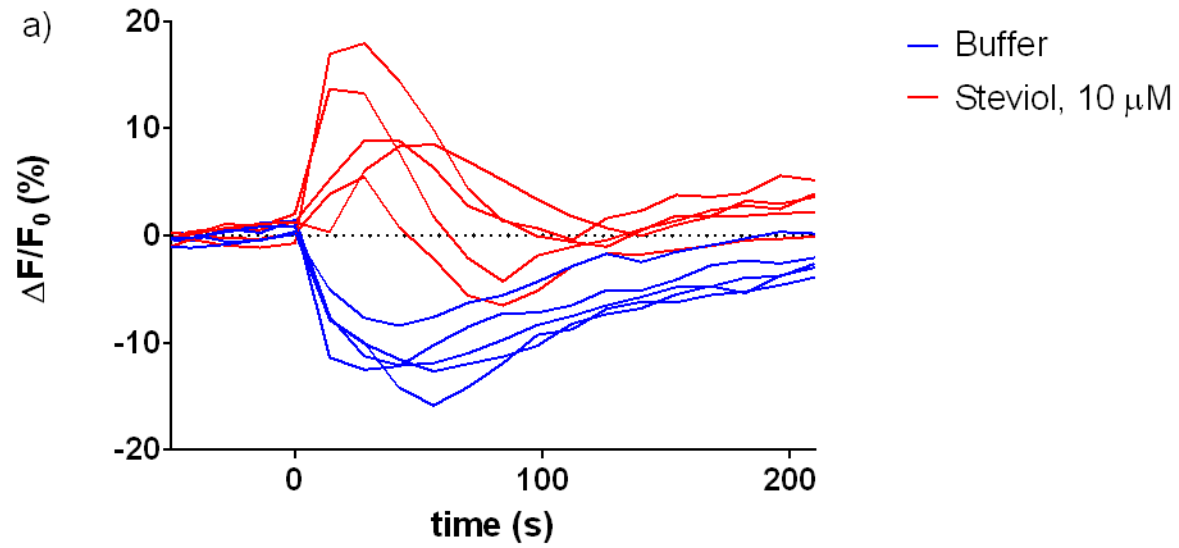


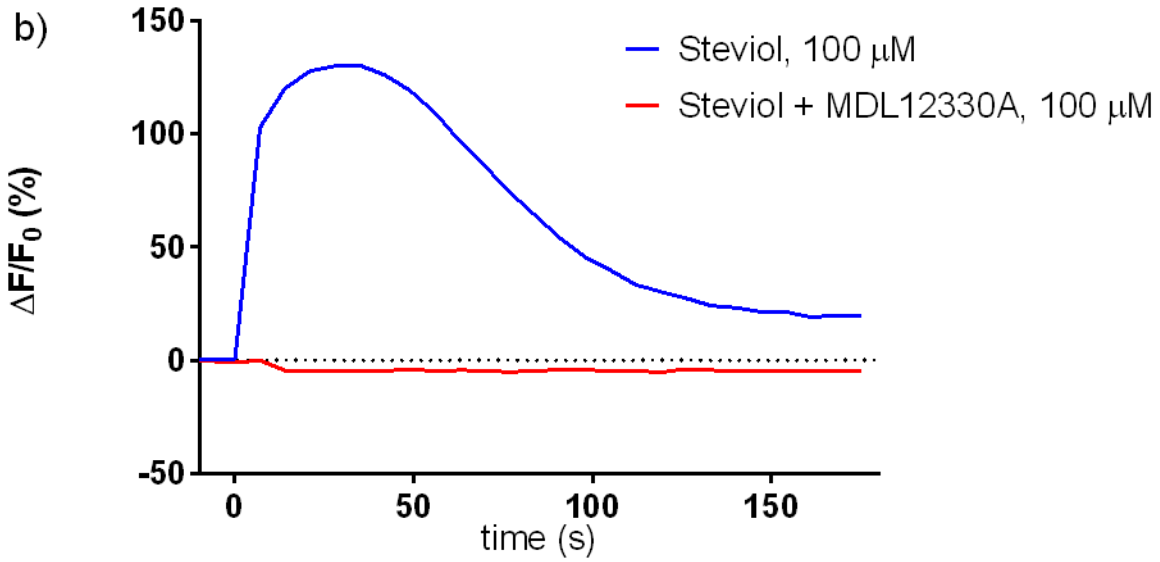
Figure 4: Progesterone production by H295R cells following exposure to 500-25,000 ng/ml steviol for 48 h. Values are means ± SD for three independent exposures in triplicate (n = 3). P = <0.05 (*) P≤ 0.001 (***).

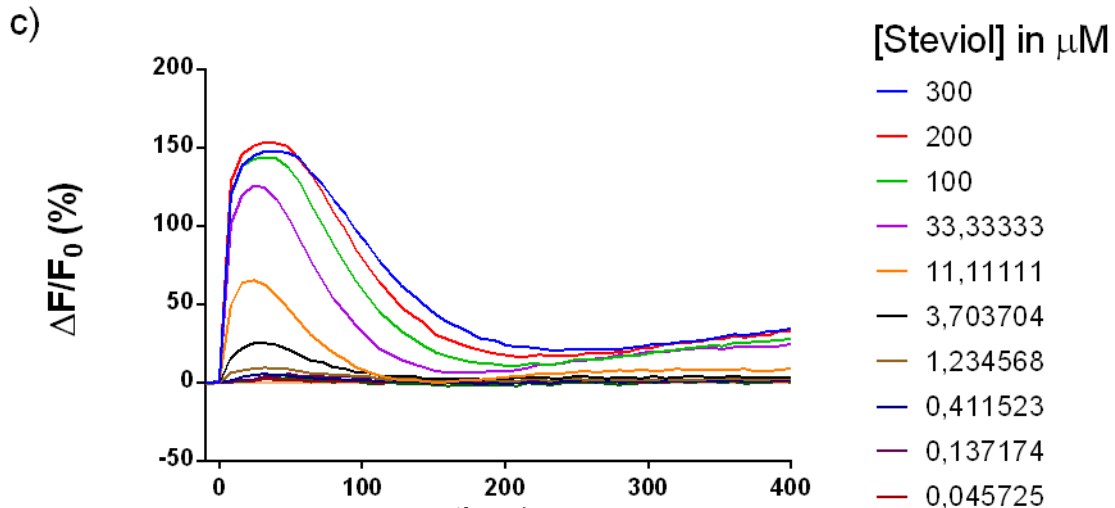
3.4 Effects on progesterone receptor of sperm, CatSper

Steviol was found to induce Ca^{2+} signals in human sperm cells (n=5) (Fig. 5a). The induced response was found to be inhibited 98.65±1.91% by the specific CatSper inhibitor MDL12330A (n=3) (Fig. 5b). Analysis of the dose-response relation yielded an EC_{50} of 10.82±1.06 µM/3,446.17±337,61 ng/ml (n=4) (Fig. 5c-d). The EC_{02} was also calculated

which corresponds to the lowest effective dose of steviol for inducing effects on CatSper, 0.676±0.623 µM/ 215.3 ± 198.4 ng/ml (n=4).







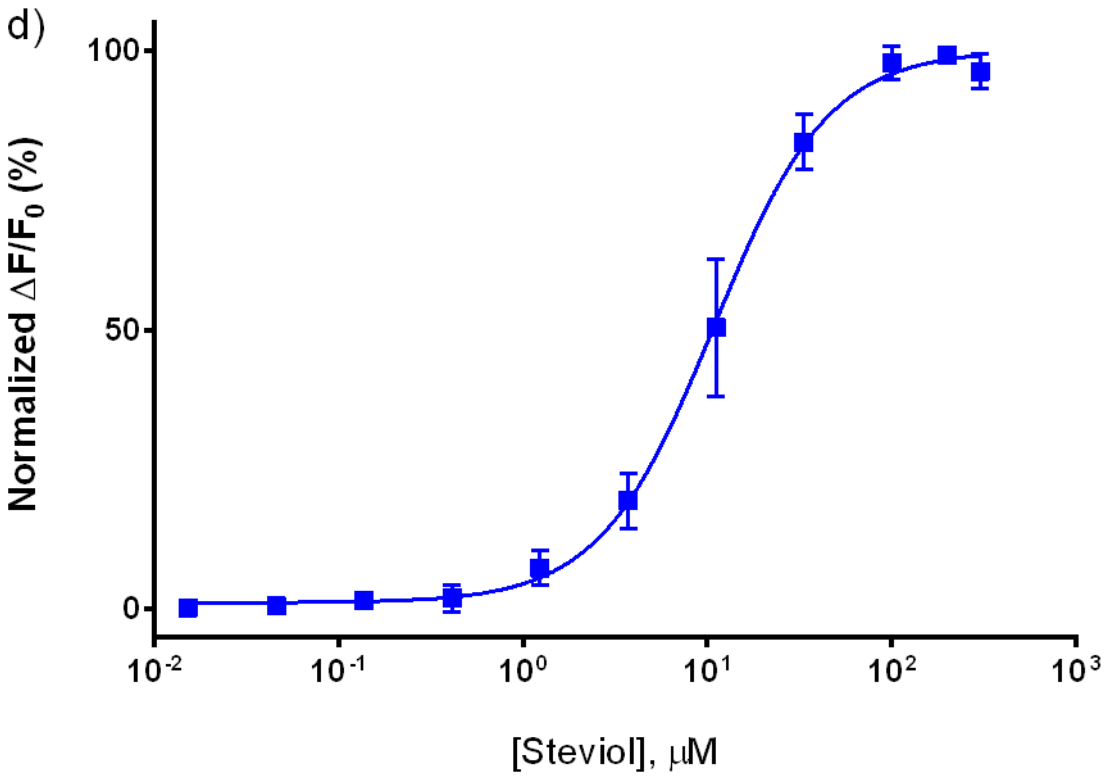


Figure 5 (a-d): (a) Ca^{2+} signals in human sperm cells induced by steviol, 10 µM, added at 0 s (n=5) (b) Steviol-induced Ca^{2+} signal in the absence (blue) and presence (red) of the CatSper inhibitor MDL12330A (c) Steviol-induced Ca^{2+} signals from one experiment, included for estimation of steviol dose-response relationship in d). (d) Normalized dose-response relationships of steviol, mean ± SEM (n=4).

4 Discussion

The endocrine disrupting effects of steviol glycosides (stevioside and rebaudioside A) and their metabolite steviol have been investigated using mammalian *in vitro* bioassays that are able to detect endocrine disruption at the level of nuclear receptor transcriptional activity and steroidogenesis. Additionally, the effect of steviol on the progesterone receptor of sperm, CatSper, was investigated.

Cell viability assays are useful controls to ensure that the effects seen in the mechanistic studies are not a result of decreasing cell viability. Cytotoxicity was observed with the higher concentrations of stevioside in the TARM-Luc (25,000 ng/ml), TM-Luc (5,000, 10,000, and 25,000 ng/ml) and TGRM-Luc (10,000 and 25,000 ng/ml) responsive RGA cell lines. Interestingly the MMV-Luc cell line did not appear to be affected by concentrations up to 25,000 ng/ml. This observed difference may be due to the fact that the parent cell line of the TARM-Luc, TM-Luc and TGRM-Luc cell lines is T47D while for MMV-Luc it is MCF-7. A similar effect was seen in a previous study by Frizzell *et al.*, (2013), where the test compound, alternariol, reduced cell viability in the three T47D cell lines but with no reduction in viability in the MMV-Luc cells (Frizzell *et.al.*,2013). Studies investigating the *in vitro* cytotoxicity of stevioside, steviol and rebaudioside A are scarce. However, Ukiya *et al.*, (2013) investigated the potential cytotoxicity of steviol in breast (SK-BR-3), leukemia (HL60), lung (A549) and stomach (AZ521) cancer cell lines by means of the MTT assay and stated that steviol at a concentration of up to 31,845 ng/ml did not induce cytotoxic effects, therefore in agreement with the present study. In spite of this, Paul et al (2012) showed a significant decrease from the solvent control at 3,185 ng/ml stevioside with the effects (71% of cells undergoing apoptosis) being more pronounced 72 h treatments. Additionally, results from the trypan blue test in this study showed that there was some cytotoxic activity occurring even at the lowest concentration tested 796 ng/ml. However, the differences in findings could be due to the fact that in the trypan blue test, cell membrane integrity is

measured rather than mitochondrial membrane integrity as measured by the MTT assay. Also in the MTT test, stevioside was exposed to the MCF-7 cells for a period of 72 h as opposed to 48 h in the current study.

Reporter gene assays provide specific and biologically relevant ways to screen substances for their hormonal effects at the level of nuclear receptor transcriptional activity (Willemsen *et al.,* 2004). The current study revealed no agonist response in any of the RGA cell lines for all compounds tested, however an antagonist response was observed in the progesterone responsive TM-Luc cell line whereby 25,000 ng/ml steviol induced a 29.1% decrease in the progesterone receptor nuclear transcriptional activity. In agreement with these findings, a study by Oliveira-Filho *et.al*., (1989) reported that the number of binding sites for androgens were not altered in rats chronically administered *Stevia rebaudiana* extract. However, effects on the androgen receptor have previously been highlighted by Uehara *et.al.,* (1982) who demonstrated that purified stevioside displaced 5a-dihydrotestosterone specifically bound to prostate androgen receptors *in vitro* (Uehara *et al.,* 1982). This finding was confirmed with the synthetic androgen, methyltrienolone, a specific ligand of androgen receptors (Uehara *et al.,* 1983). Differences in findings may be due to the varying forms of stevioside used in the studies i.e. purified or plant extract and also the type of study carried out i.e. *in vitro* vs *in vivo.*

Compounds can also be classed as EDCs through disrupting steroidogenesis; a complex process regulated by enzymes. Harvey *et al.,* (2007) state that the adrenal gland was often neglected in regulatory endocrine disruption screening and testing despite it being the most common toxicological target organ in the endocrine system (Harvey *et al.,* 2007). In the current study the H295R model was utilised to investigate the effects of steviol on steroidogenesis. It was observed that steviol had no effect on oestradiol or testosterone production; however, at 10,000 ng/ml and 25,000 ng/ml, significant increases in the

production of progesterone were observed. Progesterone is an intermediary of oestradiol and testosterone but the increased progesterone levels observed did not result in a down-stream increase of oestradiol or testosterone. However, the steroidogenesis pathway is an intricate and complicated pathway and there may be other events taking place in the pathway that are affecting the levels of testosterone and oestradiol. In addition, the H295R model has been validated as an OECD *in vitro* screening assay to screen for endocrine disruptor action on oestradiol and testosterone production. The validation process points to 48 hrs as an optimal time point with the concentrations of these hormones seemingly reaching a plateau-phase under these conditions and therefore most research to date has been carried out under these validated conditions. However, further time points, gene and protein expression studies may reveal additional effects on the levels of oestradiol and testosterone where the intermediary progesterone is increased.

Steviol was also found to induce an agonistic response on CatSper, the progesterone receptor of sperm, with an EC_{50} of 10.82±1.06 µM/3,446.17±337,61 ng/ml and an EC_{02} of 0.676±0.623 µM/ 215.3 ± 198.4 ng/ml. This response was fully inhibited (98.65±1.91%) using a specific CatSper inhibitor, indicating that the observed response is indeed mediated through direct interaction between steviol and CatSper. This is of concern because CatSper regulates several important sperm functions and is absolutely required for male fertility (Smith *et.al.,* 2013). Other studies have similiarly shown that structurally diverse chemicals can act agonistically on CatSper (Schiffer *et.al.,* 2014; Tavares *et.al.,* 2013) and that these chemicals can act additively in low doses to induce a Ca (2^+) response. This hints that even low doses in the female reproductive tract could possibly affect human sperm cell function, during their passage through the tract. To our knowledge, steviol has not been measured in reproductive system fluids. However, due to the close anatomical relationship between the distal colon/rectum with the reproductive tract in female and seminal vesicles/prostate in

447 males, it is possible that steviol absorbed in the distal colon/rectum, which bypasses the liver
448 and portal-circulation, can get into the reproductive fluids (Einer-Jensen and Hunter, 2005).

449 This study reports that steviol can modulate an increase in progesterone production and
450 antagonise the progesterone and agonise CatSper receptors. Progesterone plays a major role
451 in female reproductive health such as maintaining pregnancy, regulating the monthly
452 menstrual cycle and preparing the body for conception (Healy, 1990). Progesterone receptor
453 antagonists now have a use in clinical practice both as a contraceptive and in pregnancy
454 termination (Spitz, 2003). Planas and Kacute (1968) investigated the potential of aqueous
455 *Stevia* extract to act as a contraceptive in rats and found that the extract reduced fertility in
456 adult female rats of proven fertility and fertility continued to decrease for at least 50 to 60
457 days after intake was stopped (Planas and Kacute, 1968). A link between increased levels of
458 progesterone and weight gain has been reported (Galletti and Klopper, 1964; Lof *et al.*,
459 2009). Progesterone also has a significant role in insulin secretion. Insulin has an important
460 role in controlling blood sugar levels and therefore in patients with diabetes, insulin is either
461 at very low levels or low functioning. If insulin levels are altered, blood glucose regulation
462 will be adversely affected and subsequently, diabetes risk could increase (Diabetes UK,
463 2015). Straub *et al.*, (2001) reported that progesterone had the ability to inhibit glucose-
464 stimulated insulin secretion from isolated rat islets in a dose-dependent manner. However,
465 further in depth investigations of the relationship between progesterone levels in the body and
466 glucose-stimulated insulin secretion are required. The results of this study suggest that steviol
467 could potentially affect glucose and weight homeostasis indirectly through modulation of
468 steroid hormones. This is of heightened concern due to the fact that steviol glycosides are
469 non-calorific sweeteners aimed towards reducing the incidence of metabolic disease such as
470 diabetes and obesity.

A decrease in transcriptional activity of the progesterone receptor was observed following exposure to 25,000 ng/ml steviol. In the steroidogenesis assay, a significant increase in progesterone production was found from the lower concentration of 10,000 ng/ml steviol. A report by EFSA (2011), estimated that exposure to steviol glycosides, expressed as steviol equivalents, is around 5.6-6.8 mg/kg bw per day (5,600-6,800 ng/ml) for adult high level consumers in the UK. Additionally, for European children, anticipated exposure of the high level consumer is estimated to be 1.7-16.3 mg/kg bw per day (1,700-16,300 ng/ml). Consequently, the levels studied within this investigation are in the range of dietary exposure levels for children within the high level consumption range. Also, it is possible that as more products containing the natural sweetener *Stevia* are developed and sold, exposure levels to this compound will further increase (Goyal et al., 2009). *Stevia* will also be of particular interest to certain groups of the population with metabolic conditions such as diabetes and obesity and as a result these groups may have an even higher exposure to steviol glycosides. Therefore, there is an urgent need to ascertain the current level of exposure to steviol glycosides.

Metabolism studies in humans have reported very low blood levels of free steviol (JECFA, 2005). Steviol glucuronide has been reported to be primarily excreted in urine and steviol in faeces (Wheeler et al., 2008). As previously outlined, absorbed steviol is rapidly transformed to steviol glucuronide in the liver via conjugation to an acyl-glucuronide. Consequently, only absorbed steviol which bypasses the liver and portal-circulation, e.g. absorbed in the distal colon/rectum, will go unconjugated into general circulation and only until it passes the liver (Brusick, 2008). Studies have reported that progesterone receptors are present in the epithelial cells of the colon and progesterone has some effects directly on the gastrointestinal (GI) tract (Guarino *et.al.,* 2011; Eliakim *et.al.,* 2000). Unconjugated steviol may be present at high concentrations in the GI tract and may present the potential for steviol to inhibit progesterone

binding to the progesterone receptors. Thereby potentially enabling steviol to disturb progesterone effects in the GI tract before being conjugated in the liver. It is increasingly important that current metabolism studies are carried out to assess the metabolic pathways and effects within the framework of the growing use of stevia glycosides and a widening range of food products.

Despite the findings of this study, other research on steviol glycosides has concluded that replacing table sugar or aspartame with these natural sweeteners has various health benefits. Numerous plant glycosides have shown activity in the prevention of some dietary diseases including cancer, obesity and diabetes (Bernal *et al.,* 2011). Steviol glycosides are non-caloric and non-cariogenic sweeteners whose consumption may exert beneficial effects on human health (Gardana *et al.,* 2010).

5 Conclusion

The metabolite of steviol glycosides, steviol, can antagonise the progesterone nuclear receptor transcriptional activity and increase progesterone production. Additionally, steviol was found to induce an agonistic response on Catsper, the progesterone receptor of sperm cells. We have thus shown that steviol has the ability to affect progesterone signalling at three different sites: 1) By lowering progesterone transcriptional activity 2) by increasing the production of progesterone and 3) by acting as an agonist on Catsper, the progesterone receptor of sperm. This study highlights the endocrine disrupting potential of natural sweeteners such as those found in *Stevia rebaudiana* and suggests that emerging natural sweeteners such as *Stevia* may not be safe alternatives to sugar and other synthetic sweeteners. Further dietary exposure and metabolic studies are required to confirm their safety.

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Conflicts of interest

There are no conflicts of interest.

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