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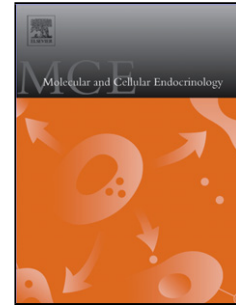
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Liquorice and glycyrrhetic acid increase DHEA and deoxycorticosterone levels *in vivo* and *in vitro* by inhibiting adrenal SULT2A1 activity

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ABSTRACT

The mineralocorticoid effects of liquorice are mediated by the inhibitory effects of one of its active components glycyrrhethinic acid on 11β -hydroxysteroid dehydrogenase type 2. However, liquorice is reputed to have many medicinal properties and also contains a number of other potentially biologically active compounds. Here we have investigated the wider effects of oral liquorice on steroidogenesis focussing particularly on possible inhibitory effects of glycyrrhethinic acid on adrenal sulfotransferase activity.

Salivary steroids were profiled by ELISA in groups of normal male and female volunteers after consuming either liquorice-containing or non-liquorice-containing confectionary for one week. Cortisol and cortisone levels reflected expected inhibition of 11β -hydroxysteroid dehydrogenase type 2 by glycyrrhethinic acid. Salivary aldosterone was decreased but deoxycorticosterone, dehydroepiandrosterone and testosterone were increased. To assess whether glycyrrhethinic acid directly affected steroidogenesis, free and conjugated steroids were measured in incubates of adrenocortical H295 cells, firstly, in the presence or absence of forskolin and, secondly, with radiolabeled deoxycorticosterone or dehydroepiandrosterone. Glycyrrhethinic acid inhibited cortisone and enhanced cortisol synthesis consistent with 11β -hydroxysteroid dehydrogenase type 2 inhibition. Basal and forskolin-stimulated synthesis of deoxycorticosterone and dehydroepiandrosterone conjugates were also inhibited in a dose-dependent manner; glycyrrhethinic acid inhibited the conjugation of deoxycorticosterone and dehydroepiandrosterone with IC_{50} values of $7\mu M$. Inhibition of deoxycorticosterone and dehydroepiandrosterone conjugation was apparent within 4h of starting glycyrrhethinic acid treatment and was not associated with changes in the expression of SULT mRNA. SULT2A1 encodes the enzyme sulfotransferase 2A1 which is responsible for the sulfonation of deoxycorticosterone and dehydroepiandrosterone as well as pregnenolone and 17-

hydroxypregnenolone in human adrenal glands. We suggest that the glycyrrhetic acid constituent of liquorice increases circulating and, thereby, salivary levels of unconjugated deoxycorticosterone and dehydroepiandrosterone by inhibiting their conjugation at source within the adrenal cortex. This effect may contribute to the mineralocorticoid actions of glycyrrhetic acid and gives substance to claims that liquorice also has androgenic properties.

Keywords: SULT2A1; DHEA; Deoxycorticosterone; Liquorice; Glycyrrhetic acid

Abbreviations

11HSD = 11 β -hydroxysteroid dehydrogenase

DOC = deoxycorticosterone

DHEA = dehydroepiandrosterone

ELISA= enzyme linked immunosorbant assay.

GA = glycyrrhetic acid

SULT2A1 = hydroxysteroid sulfotransferase 2A1

RAAS = renin-angiotensin-aldosterone system

1. Introduction

The hypertensive effects of liquorice and its active constituent glycyrrhetic acid (GA) have been widely investigated (Conn et al., 1968; Farese et al., 1991), and attributed mainly to competitive inhibition of 11 β -hydroxysteroid dehydrogenase type 2 (11HSD2) (Walker and Edwards, 1994). This enzyme is expressed in mineralocorticoid target tissues (eg colon and renal collecting duct) and normally serves to prevent glucocorticoid hormones from occupying mineralocorticoid receptors by converting biologically active cortisol to inactive cortisone. When 11HSD2 is inhibited, endogenous glucocorticoids act as mineralocorticoid hormones. However, liquorice root has been used medicinally for many years to treat a variety of conditions [eg dry cough, asthma, thirst, sterility, fever, digestive problems (Davis and Morris, 1991; Hussain, 2003)] and, more recently by the body building fraternity, to increase muscle mass (www.bodybuilder.com/store/now/lic/html; www.criticalbench.com/licorice-root-supplement-review.htm). These properties are not necessarily mediated by GA and could be due to one or more of the many other biologically active constituents of liquorice root (eg flavonoids, isoflavonoids, polysaccharides, coumarins, sterol, phytoestrogens, volatile oils, glycyramarine as well as vitamins, minerals and amino acids (Asl and Hosseinzadeh, 2008; Lee et al.; Shibata, 2000).

In addition to effects on glucocorticoid metabolism GA is thought to have other endocrine effects. For example, there have been several reports (Armanini et al., 1999; Takahashi et al., 1988; Takeuchi et al., 1991) albeit disputed (Josephs et al., 2001) that GA and glycyrrhizic acid (GZ) inhibit 17-20-lyase and 17-hydroxysteroid dehydrogenase activities thereby reducing testosterone synthesis in males and females by blocking the conversion of 17-hydroxyprogesterone (17-OHP) to androstenedione and then the conversion of androstenedione to testosterone.

Here we have gained a more comprehensive view of the effects of liquorice and GA on adrenal steroidogenesis by assaying mineralocorticoids, glucocorticoids and androgens in vivo in saliva of normal volunteers. We and others have found salivary steroid levels to be useful indicators of circulating plasma concentrations. (Atherden et al., 1985; Conway et al., 2007; Groschl, 2009). Samples can be readily obtained non-invasively from patients and volunteers without the need of supervision. The effects of GA on corticosteroidogenesis have been modelled using a multipotent human adrenocortical cell line (H295) (Rainey et al., 2004). These cells secrete aldosterone, cortisol and DHEA in response to a variety of trophic factors although responsiveness to ACTH is impaired due to an abnormal melanocortin receptor. This problem is bypassed by using forskolin to raise levels of cyclic AMP.. Based on our preliminary findings we have developed a hypothesis that GA influences circulating levels of adrenocortical derived steroid hormones by inhibiting their conjugation. In particular we have focussed on the hydroxysteroid sulfotransferase SULT2A1, an enzyme responsible for the sulfonation of DHEA, DOC and other steroids which are expressed in human adrenocortical tissue and H295 cells (Rainey et al., 2002; Strott, 2002; Kumar et al., 2009).

2. Materials and Methods

2.1. Human study

The effects of dietary liquorice on salivary steroid hormone levels were tested in groups of 10 males and 10 female volunteers in a study that was approved by Queen Margaret University's Ethical Committee. Exclusion criteria were high (systolic >140mmHg) or low (diastolic < 60mmHg) blood pressure, treatment with drugs affecting blood pressure or endocrine functions (including oral contraceptives), menstrual disorders, allergy to liquorice, and a history of kidney or liver disease.

The study followed a cross-over design to compare the effects of liquorice-containing confectionery (contents: wheat flour, glucose syrup, modified maize starch, 3% liquorice extract, caramel, salt, caramel flavouring) and non-liquorice confectionery of the same sugar content and calorific value (contents: sugar, glucose syrup, water, maize starch, beef gelatine, flavourings, cochineal colour). Volunteers were asked to consume daily 100g of non-liquorice confectionery for 1 week, followed by a wash period of 3 days, and then the liquorice confectionery for another week. Female participants were tested during the follicular phase of the menstrual cycle with day1 of the test corresponding to the start of the cycle. Body weight, height and blood pressure when lying down were recorded before and at the end of each arm of the study. Mean participant age was 23.12 ± 5.6 years and BMI was 23.26 ± 4.37 kg/m². There was no significant change in BMI with either arm of the study. Participants were given very detailed instructions on how to collect and store saliva samples. On days 7 and 10 of treatment, participants provided three saliva samples: (i) 30 minutes after waking (8.00 – 9.00h); (ii) mid-morning between 11 and 13.00h; (iii) in the evening between 19.00 and 20.00h.

2.2. Salivary steroids

Coded saliva samples were stored at -20°C prior to assay for cortisol, cortisone, aldosterone, deoxycorticosterone (DOC), testosterone and dehydroepiandrosterone (DHEA). Steroids were extracted from saliva samples with diethyl ether and measured using sensitive and specific ELISAs modified from methods previously described (Al-Dujaili 2006; Al-Dujaili et al., 2009a; Al-Dujaili et al., 2009b). Briefly, 0.5mL of saliva was vortexed with 5mL diethylether for 10min then placed in a -80C freezer. The ether was then decanted from the frozen aqueous layer into a glass tube and evaporated to dryness under nitrogen. The residue was reconstituted in 0.5mL of assay buffer. Free and conjugated levels of DHEA, DOC and other steroids were measured separately in pooled 0.5mL aliquots of male saliva after fractionation using C18 Sep-Pak cartridges (Waters Ltd, Elstree, Hertfordshire, UK). Conjugated steroids were hydrolysed prior to assay by incubation with extracts of *Helix pomatia*, then extracted with ether and assayed.

2.3. In vitro studies

NCI-H295 cells (CRL-2128, ATCC, Rockville, MD, USA) were cultured in Dulbecco's modified Eagle's medium /F12 (DMEM/F12, Invitrogen, Paisley, Renfrew, UK) supplemented with 2% cosmic calf serum (Hyclone, Thermo Scientific, Cramlington, UK), 5 µg/mL insulin, 5 µg/mL transferrin and 5 ng/mL sodium selenite (as 1% ITS, Invitrogen) at 37°C with 5% CO₂-95% air. Cells were seeded at 500,000 cells/well in 6-well plates. For experiments, cells were serum-deprived overnight and incubated for up to 24h with various concentrations of glycyrrhetic acid (0-50µM) in the presence or absence of forskolin (10 mM). In some experiments steroid conjugation was measured directly by incubating cells

with [³H]-DHEA (GE Healthcare, Amersham Bucks, UK) and [³H]-DOC (MP Biomedicals, Solon, Ohio, USA). The medium was removed and stored at -80°C for steroid analysis; remaining cells were lysed and RNA extracted using RNeasy mini-kits (Qiagen, Crawley, West Sussex, UK). Steroids were extracted from the medium using C18 Sep-Pak cartridges; conjugated and free steroids were eluted separately with 40% and 100% aqueous methanol respectively. Radioactivity in free and conjugated fractions was measured by scintillation counting. Absolute levels of steroids were measured by ELISA; conjugated steroids were hydrolysed with *Helix pomatia* prior to assay.

2.4. Real time PCR assays

RNA was extracted using Qiagen RNeasy Mini kit and genomic DNA was excluded by DNase treatment of samples, on-column, with the RNase free DNase set (Qiagen) according to supplier's protocol. RNA purity and quantification were assessed spectrophotometrically (Nanodrop model ND-1000, Nanodrop Technologies Inc, Wilmington, DE, USA). mRNA (200ng) was reversed transcribed using RT-Reagent kits (Applied Biosystems, Applera, Warrington, Cheshire, UK). Real-Time PCR was performed for SULT2A1 cDNA and ribosomal 18S RNA using pre-validated Assay-on-Demand System reagents (Applied Biosystems). Briefly, 2µL cDNA was used as a template mixed with 1X universal Taqman master cocktail (Applied Biosystems) and the specific set of 1X primer/probe mixture. Duplicate reactions in 96-well plates were evaluated using an ABI Prism 7900 Sequence Detector (Applied Biosystems). The C_T value obtained for the 18S species was used to confirm cDNA quality and as an internal reference control for SULT2A1 expression. 18S RNA has been used routinely in our experiments with H295 cells over several years and has proved to be very reliable for quality control and normalisation purposes.

2.5. Statistics

For salivary steroids the sex-dependent and daytime variations of the effects of liquorice containing and non-liquorice containing confectionery were compared using a general linear model of analysis of variance. Data for the effects of confectionery were matched for each individual. For studies of steroid synthesis and SULT2A1 expression in H295 cells, the effects of forskolin and GA were tested by analysis of variance with Neuman-Keul's correction. Results are expressed as mean values \pm SE and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of liquorice on salivary steroid concentration

There was no significant increase in systolic or diastolic blood pressure due to 1 week consumption of liquorice confectionery. Figure 1 and Table 1 shows that the overall effect of liquorice on salivary steroid concentrations in males and females is broadly similar. Cortisol, deoxycorticosterone, DHEA and testosterone were increased (although not significantly for cortisol in males) whereas aldosterone and cortisone were decreased. Effects on cortisol and cortisone are in line with known inhibition of 11β -hydroxysteroid dehydrogenase by liquorice and GA which are known to cause hypertension via a mineralocorticoid-dependent process. Apparent mineralocorticoid excess due to cortisol occupancy of MR is reflected by decreased aldosterone levels.

Increased DHEA and testosterone levels in response to liquorice were not expected. These effects are more prominent in the evening when cortisone levels are least affected (figure 2). Potentially testosterone increases could arise from peripheral conversion of DHEA derived from the adrenal gland. However, salivary testosterone and DHEA concentrations were

significantly correlated only in males ($r = 0.4$, $P = 0.002$; females: $r = 0.2$, $P = 0.1$). Increases in deoxycorticosterone, DHEA and cortisol could be explained by effects of liquorice on adrenocortical steroidogenesis, particularly as their routes of metabolism are different.

Table 2 shows the effects of liquorice on free and conjugated steroids in pooled saliva from male subjects. The pattern of fractionated free steroid values was similar to that of unfractionated steroid values. Levels of DHEA and DOC conjugates were decreased by liquorice treatment; other steroid conjugates were not significantly affected.

3.2. Effects of GA on basal and forskolin-stimulated steroidogenesis by H295 cells

Responsiveness to ACTH is compromised in H295 cells although cyclic AMP-dependent signalling is preserved. Forskolin stimulates adenyl cyclase independent of ACTH receptors and was used in the present studies to stimulate steroidogenesis via cyclic AMP. Figure 3 shows that synthesis of unconjugated steroids were all stimulated by forskolin (DHEA > cortisol > cortisone > deoxycorticosterone > aldosterone). Similar results were obtained with vasoactive intestinal peptide, an agonist with activity which is not dependent on cyclic AMP (data not shown). As expected of an adrenocortical cell line, very low levels of testosterone were detected in the medium and were unresponsive to forskolin treatment (data not shown). GA reduced both basal and forskolin-stimulated cortisone synthesis ($P < 0.01$) but did not significantly affect cortisol synthesis. Basal and forskolin-stimulated DOC and DHEA synthesis were increased by GA but only effects on basal synthesis were statistically significant ($P < 0.01$). Aldosterone responses to GA were not statistically significant.

3.3. Dose dependent effects of GA on conjugation of steroids synthesized by H295 cells

In the absence of GA similar amounts of DHEA and DHEAS were released by H295 cells; the degree of conjugation was not affected by forskolin (figure 4). DOC conjugation was

approximately half that of DHEA and, in the absence of GA was slightly higher in forskolin-stimulated cells. Cortisol, cortisone and aldosterone conjugation was 9.9, 30.0 and 62% respectively in unstimulated cells and was reduced by forskolin treatment (3.3, 15 and 45%) but not by GA either in the presence or absence of forskolin. GA caused dose-dependent decreases in the degree of DOC and DHEA conjugation in both basal and forskolin-stimulated cells. Non-conjugated DHEA and especially DOC were increased by low doses of GA ($\leq 10\mu\text{M}$) commensurate with decreased conjugation. However, the total amounts of DHEA released (conjugated plus unconjugated) were inhibited by high doses of GA ($\geq 25\mu\text{M}$).

3.4. GA inhibits 11β -hydroxysteroid dehydrogenase activity in H295 cells

Cortisone was markedly inhibited by GA (figure 5). The ratio of cortisone:cortisol was decreased by the lowest dose of GA ($1\mu\text{M}$) in forskolin stimulated cells ($P=0.01$) indicating that 11β -hydroxysteroid dehydrogenase activity is more sensitive to the inhibitory effects of GA than enzymes responsible for conjugation. However, the net effect of GA on cortisol synthesis due to $11\text{HSD}2$ inhibition is probably negligible since, in the absence of GA, the proportion of cortisol converted to cortisone is less than 3% for unstimulated cells and less than 1% for forskolin-stimulated cells.

3.5. GA inhibits conjugation of radiolabeled DOC and DHEA

To confirm that GA inhibits conjugation of DOC and DHEA rather than de novo synthesis of steroids, H295 cells were incubated with either ^3H -DHEA or ^3H -DOC. Conjugation of both steroids was inhibited partially with $3\mu\text{M}$ GA and by more than 80% with $30\mu\text{M}$ GA (figure 6). The inhibitory effects of the higher dose of GA were apparent within 4h (figure 7).

3.6. GA effects on conjugation are not due to inhibition of SULT2A1 gene transcription

Previous studies have identified DOC and DHEA sulfates as conjugates that are derived principally from the adrenal gland due to the specific expression of SULT2A1. To establish whether GA effects on DOC and DHEA conjugation were due to inhibition of transcription, SULT2A1 mRNA transcript levels were measured by quantitative RT-PCR in cells treated with GA. Despite evidence of marked inhibition of steroid conjugation, SULT2A1 mRNA levels were unaffected (figure 7), suggesting that GA directly affects SULT2A1 enzyme activity.

4. Discussion

The effects of liquorice on salivary steroid levels are, in part, explained by the known inhibitory effects of glycyrrhetic acid (GA) on 11β -hydroxysteroid dehydrogenase activity (Conn et al., 1968; Farese et al., 1991; Walker and Edwards, 1994). The hypertensinogenic response to liquorice is due to a block in the conversion of active endogenous glucocorticoid hormone, cortisol, to inactive cortisone. The isoform of the enzyme responsible for this conversion, 11β HSD2, is expressed in mineralocorticoid responsive target tissues where it serves to prevent high concentration of circulating glucocorticoid hormones from binding inappropriately to mineralocorticoid receptors (MR). When 11β HSD2 is inhibited, as shown by marked decrease in salivary cortisone:cortisol ratios, then glucocorticoids can cause MR-dependent sodium retention, plasma volume expansion and ultimately an increase in blood pressure (Quinkler and Stewart 2003; Walker and Edwards, 1994). In the present study, liquorice did not cause hypertension perhaps because the period of treatment was too short or the dose of liquorice too small (Sigurjonsdottir et al., 2001). However, there was a decrease

in salivary aldosterone, an indication of the suppression of the renin-angiotensin-aldosterone system (RAAS) when endogenous glucocorticoids activate MR. It should be noted that GA did not inhibit aldosterone synthesis by H295 cells indicating that aldosterone decreases *in vivo* are secondary to mineralocorticoid excess caused by other steroids. The ratio of cortisone:cortisol synthesised by H295 cells is low (<0.02) and was reduced even further by GA. Assuming that endogenous adrenocortical production similarly favours cortisol over cortisone, it seems likely that the much higher cortisone to cortisol ratios in saliva reflect the effects of GA on extra-adrenal glucocorticoid metabolism.

Liquorice also caused increases in salivary DOC, DHEA and testosterone which may have additional pharmacological consequences. Firstly DOC is recognised as a weak mineralocorticoid and could, therefore, contribute to hypertensinogenic processes and suppression of RAAS. Secondly, increased DHEA and testosterone could have androgenic effects. This observation supports claims that liquorice promotes increases in muscle mass (www.bodybuilder.com/store/now/lic/html; www.criticalbench.com/licorice-root-supplement-review.htm). It should be noted, however, that others have reported that GA binds to the androgen receptor and could, therefore, exert more direct effects (Tamaya et al., 1986).

The mechanism responsible for increases in DOC, DHEA and testosterone are not immediately apparent. None of them could be considered substrates for 11HSD2 although androgen and DOC metabolites are antagonists of 11HSD activity (Morris et al., 2007). Three possible explanations are suggested: (i) GA has been shown to inhibit two hepatic enzymes (3β -hydroxysteroid dehydrogenase and steroid 5β reductase) responsible for ring A reduction of steroid hormones (Latif et al., 1990). This could prolong the plasma half life of DOC and testosterone. However, aldosterone and cortisone are also catabolised by this route yet are present at lower not higher concentrations in saliva after liquorice treatment; (ii) GA could affect de novo androgen biosynthesis. It has been suggested that 11HSD1 activity modulates

the inhibitory effects of glucocorticoids on testosterone synthesis by the Leydig cells of the testis (Hu et al., 2008; Monder et al., 1994). If GA were to inhibit the capacity of 11HSD1 to regenerate active glucocorticoid hormone, this would lessen the effects of glucocorticoids on androgen production. Against this hypothesis is the fact that the 11HSD1 enzyme is less susceptible to GA inhibition than 11HSD2 (Latif et al., 1997; Marandici and Monder, 1993) and is thought to contribute to dehydrogenase activity in Leydig cells (Ge et al., 2005). Also in the present study, effects of liquorice on salivary androgens were seen in both males and females in the presence of higher cortisol levels; (iii) GA may directly affect adrenal androgen production. In primates, DHEA and its sulfate are derived predominantly from the adrenal gland with males producing slightly more than females (Labrie et al., 2003). DHEA is converted to testosterone by peripheral tissues. To test this third possibility, we measured steroidogenesis in H295 cells treated with GA. H295 cells are a multipotent adrenal cell line which synthesise DHEA, DOC, cortisol and cortisone and aldosterone. A preliminary study confirmed that the profile of steroids by these cells in response to GA (figure 3) was similar to those of saliva after feeding liquorice except that testosterone levels were very low and unresponsive to trophic factors and did not respond consistently to GA. We were aware however, that GA induced changes reflected free steroid levels and did not take account of the very high levels of conjugated steroids that are synthesised by the adrenal cortex and by H295 cells. In particular, it is known that pregnenolone, 17-hydroxypregnenolone, deoxycorticosterone and DHEA are secreted as sulfates (Rainey et al., 2002; Strott, 2002).

The effects of GA on conjugated and free steroid production were tested in basal and stimulated H295 cells. Conjugated DHEA and DOC were decreased in a dose dependent manner whereas free DHEA and DOC were increased by 10 μ M GA. In contrast, the increase in total cortisol caused by 10 μ M GA was related to inhibition of 11HSD activity since cortisone was decreased and cortisol conjugation was unaffected. High doses of GA (>

50 μ M) tended to reduce synthesis of total DHEA and increases in DOC and cortisol appeared to have reached a plateau. Arguably this high dose effect could be a non-specific toxic effect although there were no signs of cell death. An alternative possibility is that GA inhibits the sulfation of pregnenolone, a known substrate of adrenal sulfotransferase activity and an early intermediate in glucocorticoid biosynthesis. This would shunt steroidogenesis towards the synthesis of DOC and could also contribute to the amounts of basal and forskolin stimulated total cortisol release that were increased by GA (figure 5).

Previous studies have identified DOC and DHEA conjugates in the circulation as sulates. Both liver and adrenal glands express SULT2A1, an enzyme that has the activity to sulfonate both steroids (Richies et al, 2009). However, tracer studies indicate these sulates are synthesised by the human adrenal gland rather than the liver (Casey and MacDonald, 1983; Labrie, 1991); hepatic SULT2A1 appears to be responsible for the disposal of xenobiotics (Senggunpai, Yoshina and Yamazoe, 2009). The known specificity of SULT2A1 and its expression in H295 cells indicate that this enzyme is responsible for DOC and DHEA sulfation in these cells and is therefore a likely target of GA. The inhibition of steroid sulfation by GA appears to be a direct effect on enzyme activity rather than a decrease in SULT2A1 gene expression. Conjugation of radiolabeled DOC and DHEA was reduced 8h after addition of GA at a concentration which did not affect SULT2A1 mRNA expression. Others have also reported a disparity between SULT2A1 activity and gene expression and in H295 cells. Kumar et al (2009) showed that testosterone and insulin treatment caused a marked inhibition of DHEA sulfation despite a three fold increase in SULT2A1 expression. In our experiments we suggest that GA is a competitive inhibitor of SULT2A1 activity and its effects are therefore independent of transcriptional or translational control.

The effects of GA *in vitro* on H295 cells may differ from those expected in the adrenal gland *in vivo*. SULT2A1 is uniformly expressed by H295 cells so that an increase in free steroid availability caused by inhibition of conjugation will provide substrate for alternative competing enzymes. In contrast adrenal SULT2A1 levels vary at different stages of life and are localised to specific regions of the cortex (Rainey et al., 2002). In adulthood, for example, plasma DHEAS levels are highest at age 20-30 years and SULT2A1 expression are localised predominantly in the zona reticularis. Given that mineralocorticoids, glucocorticoids and androgens are produced by different zones of the cortex, it follows that GA effects on salivary steroid levels may reflect zone-specific effects on different steroidogenic enzymes

5. Conclusions

We have demonstrated that liquorice increases salivary DHEA, DOC and testosterone levels as well as showing expected inhibition of cortisol to cortisone conversion. *In vitro* analysis of steroidogenesis by H295 cells treated with GA show that *in vivo* DHEA and DOC effects are probably due to inhibition of steroid conjugation by the adrenal gland. Although these GA effects are not caused by changes in the transcription of SULT2A1 (the enzyme responsible for DOC and DHEA sulfation), it seems clear that the control of SULT2A1 activity, whether genomic or non-genomic, plays an important part in directing pathways of mineralocorticoid, glucocorticoid and androgen biosynthesis.

Disclosure statement

The authors have nothing to disclose.

Conflict of interest statement

The authors have declared no conflict of interest.

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Steroid	Liquorice treatment	Sample Time	Sex	Treatment *Sample time	Treatment *Sex
DHEA	0.0005	0.4	0.01	0.02	0.9
DOC	0.0005	0.4	0.0005	0.4	0.7
Cortisol	0.0005	0.2	0.0005	0.7	0.001
Cortisone	0.03	0.0005	0.001	0.2	0.8
Aldosterone	0.03	0.93	0.001	0.8	0.5
Testosterone	0.0005	0.06	0.0005	0.1	0.7

Table 1. Probability values for effects of liquorice, time of sampling and sex on salivary steroid levels. Also shown are values for interactions between treatment and sample time and treatment and sex. There were no significant differences for a three way interaction between treatment, sample time and sex.

Steroid	Free Fraction (nM)			Conjugated Fraction (nM)		
	Non-liquorice	Liquorice	P value	Non-liquorice	Liquorice	P value
DHEA	7.6±3.4	17.6±5.5	0.001	2.2±0.6	1.3±0.3	0.03
DOC	0.23±0.04	0.32±0.05	0.02	0.75±0.08	0.6±0.09	0.01
Cortisol	17.8±2.8	22.9±3.3	0.01	10.7±1.9	12.2±1.6	0.2
Cortisone	0.62±0.12	0.34±0.06	0.01	0.15±0.04	0.13±0.03	0.6
Aldosterone	0.11±0.01	0.06±0.01	0.02	0.08±0.01	0.07±0.01	0.2
Testosterone	0.82±0.08	1.03±0.14	0.05	2.39±0.33	2.61±0.05	0.3

Table 2. Effects of liquorice on free and conjugated salivary steroid levels from male volunteers. Saliva samples (0.5ml) collected at 09.00, 12.00 and 19.00h were pooled and fractionated with Sep-Pak C18 cartridges as described in the methods section. Conjugated steroids were hydrolysed with *Helix pomatia* prior to assay. Results are means ± SEM.

Figure Legends

Figure 1: Changes in salivary steroid levels in men and women in response to liquorice.

Values shown are means \pm SEM of the percentage change in salivary steroid levels after feeding liquorice to groups of ten men (open bars) and ten women (solid bars) compared with pre-treatment control values. Values for each individual from samples collected on three separate occasions have been averaged for the non-liquorice and for the liquorice arms of the study. Average values with liquorice treatment are expressed as a percentage of average non-liquorice values. Significant effects of liquorice are indicated as * - $P < 0.05$; ** - $P < 0.01$, *** - $P < 0.001$.

Figure 2: Salivary steroid levels throughout the day with non-liquorice confectionery (open circles) and liquorice confectionery (solid circles). Values shown are means \pm SEM of twenty volunteers (10 men, 10 women).

Figure 3: Effects of glycyrrhetic acid on steroidogenesis in H295 cells incubated with and without forskolin. Medium from H295 cells cultured for 24h with and without 10 μ M GA and with and without 10mM forskolin were analysed for unconjugated steroid hormone levels by ELISA. Results (n = 4, mean \pm SEM) are expressed as fold differences relative to controls.

Figure 4: Concentration-dependent effects of glycyrrhetic acid on DOC and DHEA conjugation by H295 cells incubated with (open circles) and without (solid circles) forskolin. Results are measurements of unconjugated and conjugated DOC and DHEA levels in culture medium of H295 cells incubated for 24h with and without 10mM forskolin in the

presence of increasing concentrations of GA (n = 4, mean \pm SEM). Percentage conjugation = % (conjugate) / (conjugate + unconjugated) values.

Figure 5: Concentration-dependent effects of glycyrrhetic acid on 11 β -hydroxysteroid dehydrogenase activity. Results are measurements of total (unconjugated + conjugated) levels in culture medium of H295 cells incubated for 24h with and without 10mM forskolin in the presence of increasing concentrations of GA (n = 4, mean \pm SEM). The ratio of total cortisone: total cortisol is taken as an index of net 11 β -hydroxysteroid dehydrogenase activity.

Figure 6: Concentration-dependent inhibition of radiolabeled DOC and DHEA conjugation by glycyrrhetic acid. H295 cells were incubated for 24h with ^3H DOC or ^3H DHEA in the presence of increasing concentrations of GA. The culture medium of each sample was fractionated using Sep-Pak C18 cartridges and radioactivity associated with free or conjugated fractions was counted. Percentage conjugation = % (radioactivity of conjugate fraction) / (radioactivity of conjugate + unconjugate fractions). Results are means \pm SEM, n=4. Significant effects of GA compared with controls are indicated as * -P<0.05; ** -P<0.01.

Figure 7: Temporal effects of glycyrrhetic acid on DHEA conjugation and SULT2A1 mRNA expression by H295 cells. DHEA conjugation was measured using radiolabeled DHEA (see figure 6) in medium collected at intervals after addition of 30 μM glycyrrhetic acid to H295 cells. RNA extracted from cells at these time points were used to quantify SULT2A1 mRNA (n = 4, mean \pm SEM) by real time RTPCR with results normalised with

18S RNA levels. Significant effects of GA compared with controls are indicated as * -
P<0.05; ** -P<0.01.

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Figure 1

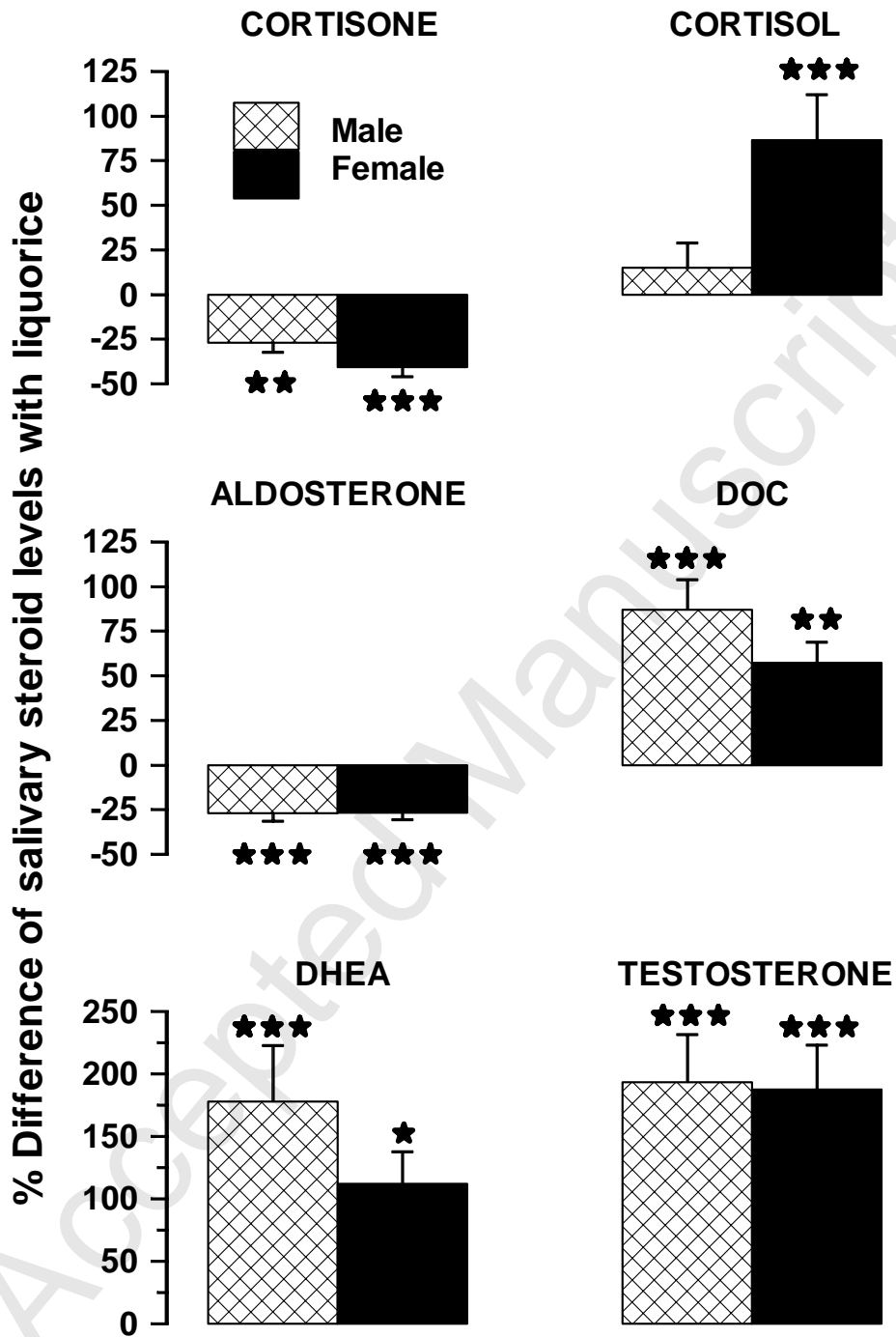


Figure 2

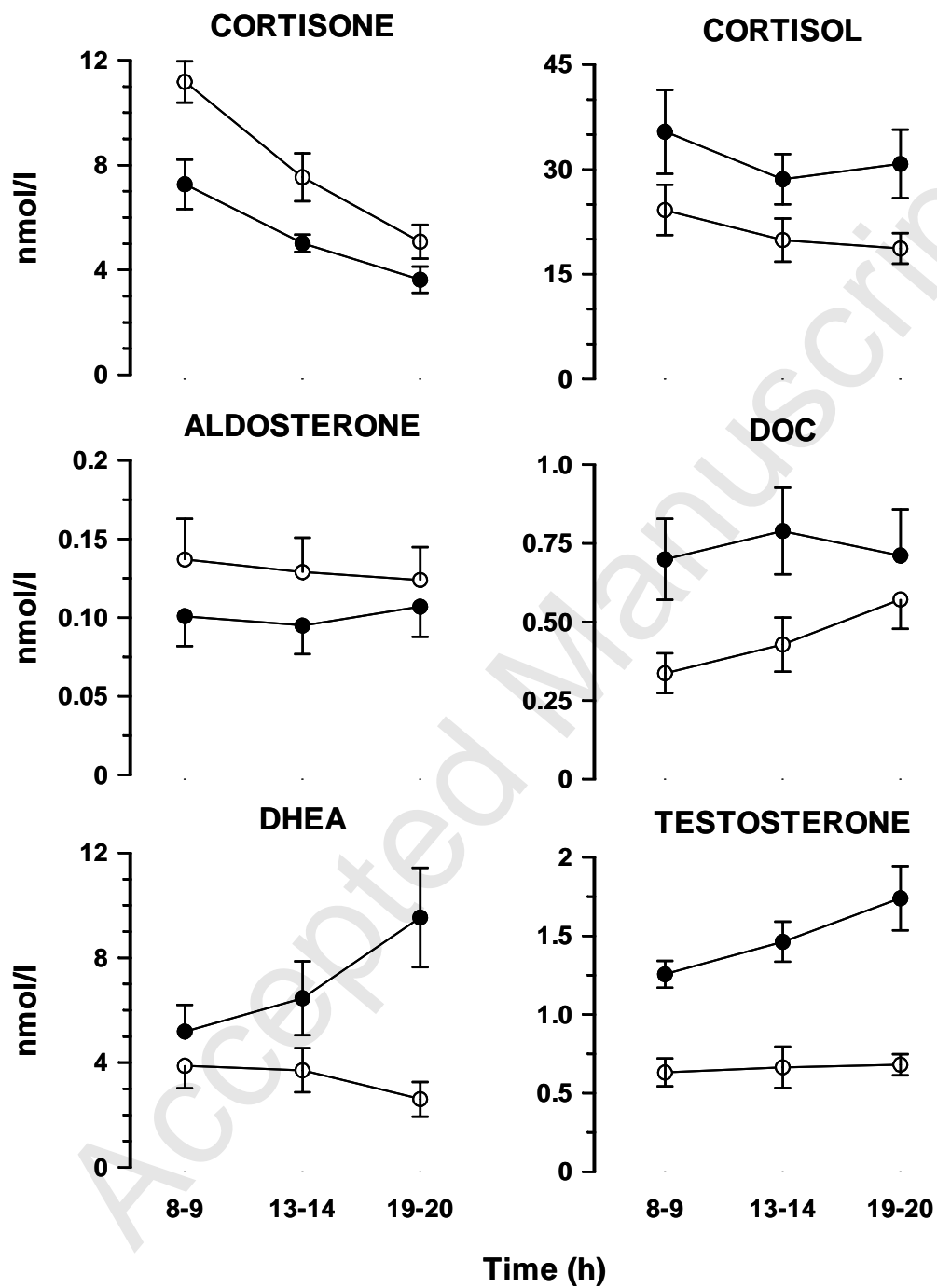


Figure 3

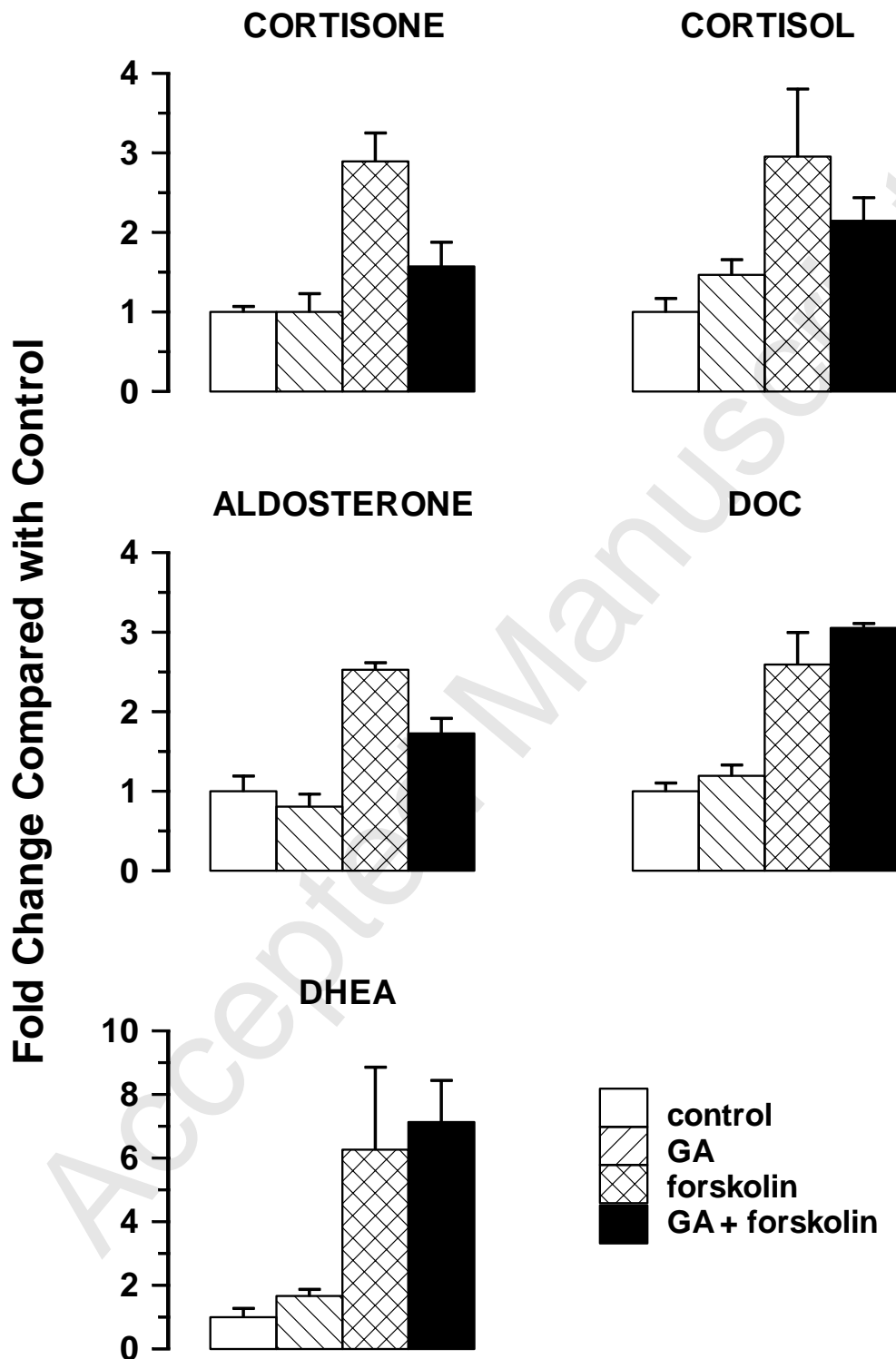


Figure 4

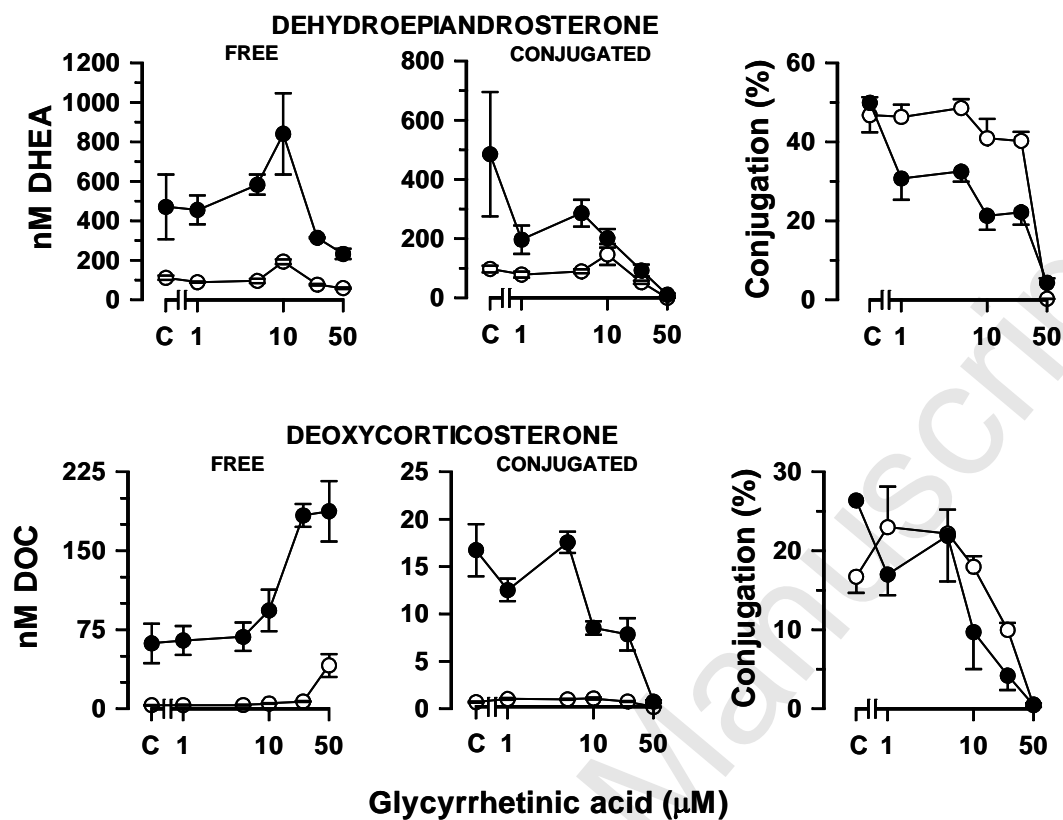


Figure 5

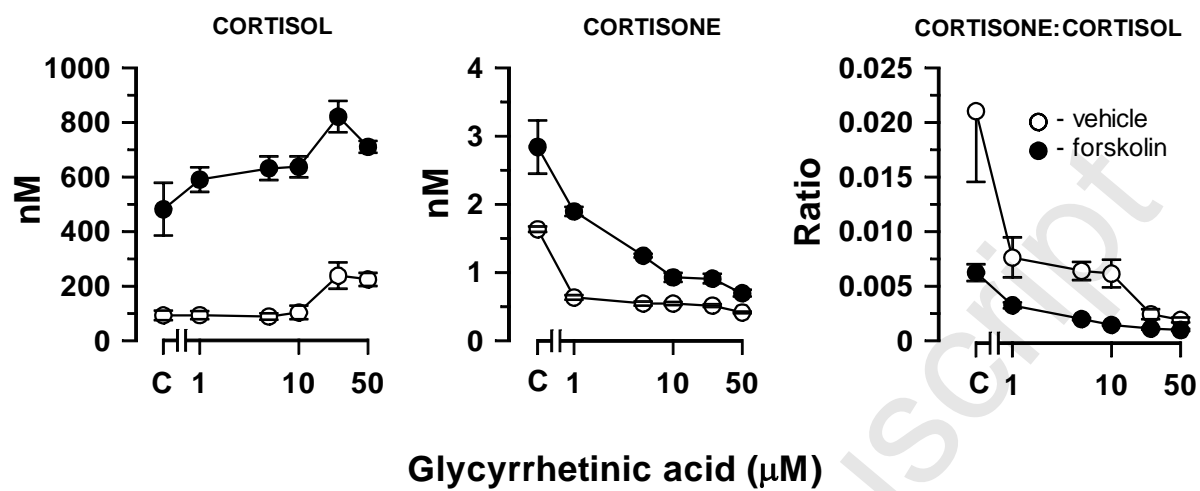


Figure 6

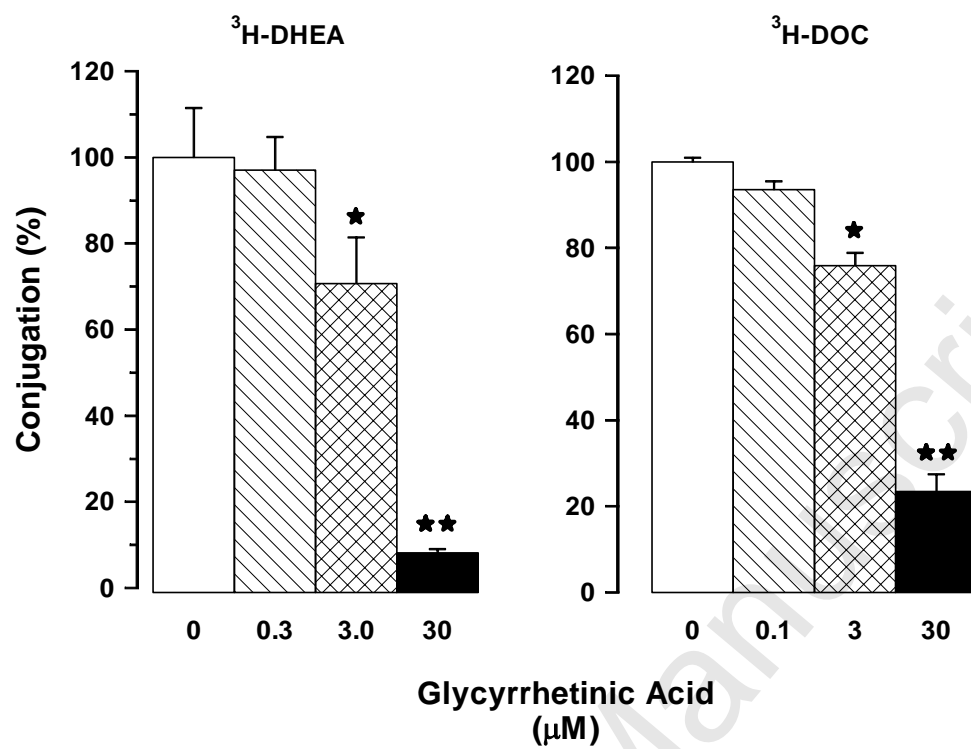


Figure 7

