
Salivary or Serum Cortisol: Possible Implications for Alcohol Research

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<http://dx.doi.org/10.5772/51436>

1. Introduction

Alcohol consumption can induce the development of nutritional disorders as alcohol ingestion often replaces food intake [1]. The long-term intake of alcohol decreases the amount of food consumed when food is freely available [2], and the degree of malnutrition may be related to the irregularity of feeding habits and intensity of alcohol intake [3]. The repercussions of alcohol abuse (over time) can involve damage to most of the major organs and systems in the body [4]. However, despite the overwhelming evidence linking alcohol to ill health the role (if any) alcohol plays in the development of disease remains uncertain.

The hypothalamic-pituitary-adrenal (HPA) axis is responsible for the synthesis and release of steroid hormones, the most abundant being dehydroepiandrosterone (DHEA), DHEA sulfate (DHEAS), cortisol, and aldosterone [e.g. 5]. The release of either corticotropin-releasing factor or arginine vasopressin by the hypothalamus stimulates the anterior pituitary to release adrenocorticotropin (ACTH), which promotes the synthesis and release of steroid hormones that have glucocorticoid (i.e. cortisol), mineralocorticoid (i.e. aldosterone), and androgenic (i.e. DHEA, DHEAS) functions [6].

Steroid hormones have a diverse and highly important role in the body and any dysregulation in steroid activity can lead to the development of disease. The adrenocortical system is markedly altered by food availability and an elevation in cortisol is commonly observed under fasting conditions [7-9]. Cortisol plays a major role in the regulation of carbohydrate, protein, and lipid metabolism [10,11] and during prolonged fasting by stimulating gluconeogenesis acts to protect the body from cellular damage until food once again becomes available [7,8,10-14].

It is well accepted that alcohol consumption can significantly reduce DHEAS [9, 15] and aldosterone [16, 17]. However, the literature is highly contradictory with respect to the effect of oral alcohol intake on cortisol. Investigations have shown that blood alcohol concentrations exceeding 1 g/L can elevate plasma cortisol [18]. Moreover, it has also been reported that low to moderate alcohol intake has little [19], if any effect on [20-24] or may even significantly reduce [9], cortisol concentration. Furthermore, early work showed that while alcohol consumption may promote a significant decrease in cortisol (initially) this is later followed by a significant elevation in plasma cortisol concentration [25].

In the past it has been proposed that the discrepancy in cortisol, noted in the alcohol literature, could be due to differences in stress levels associated with the testing procedure [26]. Cortisol is rapidly released in response to stress and the stress associated with blood sampling (alone) can falsely increase cortisol values during the study. Furthermore, as each individual responds differently to the blood taking procedure, the difference in stress levels between individuals may be responsible for the discrepancy in findings observed between studies employing similar blood sampling methodology [27].

Plasma free cortisol measurement is the most reliable measure of adrenal glucocorticoid activity as plasma total cortisol values may be affected by the alteration of its carrier protein, CBG [28]. However, it is not unusual in psychoneuroendocrinology for the assessment of cortisol to be made in saliva [29], as the amount of cortisol in saliva is highly correlated with the level of plasma free cortisol [8,30-32], and the level of cortisol in cerebrospinal fluid (CSF). Moreover, steroid hormone concentration in saliva is not dependent on saliva flow rate and no dilution effect has been observed [33]. Therefore, as cortisol is released in response to stress [34], salivary assessments of cortisol, due to the non-invasive nature of the sampling procedure, may provide a more reliable measure of steroid activity.

The aim of this study was to clarify the effect (if any) of consuming a small-moderate amount of white wine on cortisol by comparing the effect (if any) of consuming a small-moderate amount of white wine on salivary cortisol and serum cortisol, and salivary cortisol alone.

2. Method

2.1. Subjects

A total of 16 subjects aged 19-22 years were recruited to participate in one of two alcohol trials. Eight subjects were recruited on two separate occasions. Due to early suggestions that under conditions of stress cortisol release may be influenced by gender factors [35] only males were recruited.

Subjects were excluded if they reported to have had: a previous history of psychiatric disorder; any neurological disease; any major physical complaint, including Type 1 or Type 2 diabetes; a history of drug use; taken any prescribed medication within the last 7 days; routinely engaged in shift work; or satisfied the DSM-IV-TR diagnostic criteria for alcohol abuse and/or dependence (American Psychiatric Association, 2000).

Individuals were all white Caucasians of Australian or British origin. The height of subjects in both trials was 175-182cm. No subject was obese as the weight of all subjects when assessed was within the medically recommended range for age and height.

The majority of subjects lived at home with family ($n = 11$) while the remainder lived in shared accommodation ($n = 5$). None of the subjects reported to have a family history of alcoholism and the group of 16 males contained only one non-drinker. The age subjects first consumed alcohol was reportedly between 14.4 years and 17 years. A little less than 40% regularly consumed a mixture of alcoholic beverages ($n = 6$), while others preferred to drink beer ($n = 7$) or spirits ($n = 2$), only.

Subject participation was obtained by informed consent. Approval for the study was granted by La Trobe University Human Ethics Committee who determined that the procedures were consistent with ethical guidelines for human research set by the National Health and Medical Research Council of Australia.

2.2. Equipment and assays

Assessment of cortisol in saliva was made using COBAS ELECSYS 2010 immunoassay (Roche Diagnostics, Indianapolis, IN, USA). The intra-assay coefficient of variation (CV) was 6.1% at 4.68 nmol/L, 2.7% at 11.5 nmol/L, 1.5% at 15.9 nmol/L, and 2.8% at 19.8 nmol/L. Quality controls for the assessment of cortisol in saliva was performed using Bio Rad Unassayed Liquicheck Chemistry Control (Bio Rad, USA, Lot # 16340 Expiry date: 12/2007). Staff at Analytical Reference Laboratories (St. Kilda Road, Melbourne, Australia), who were blind to the experimental manipulations, performed the cortisol analyses.

Serum samples were assayed locally to determine free cortisol levels using IBL Cortisol ELISA kits (RE52061, IBL Gesellschaft Für Immunchemie Und Immunbiologie MBH, Flughafenstrasse 52a, D-22335 Hamburg, Germany). The intra-assay CV of this coated-well competitive binding radioimmunoassay was 8.1% at 43.5 ng/ml, 3.2% at 226.5 ng/ml, and 5.6 % at 403.6 ng/ml. The inter-assay CV was 6.6% at 55 ng/ml, 7.7% at 209 ng/ml, and 6.5% at 361 ng/ml.

Semi-quantitative urinalysis was performed using Labstix™ (Bayer Australia Limited), in order to measure ketones (sensitivity was 0.5-1.0 mmol/l acetoacetic acid), glucose (sensitivity was 4-7 mmol/l glucose), blood (sensitivity was 150-620 µg/l haemoglobin), protein (sensitivity was 0.15-0.30 g/l albumin), and pH. Blood alcohol level (BAL) was assessed using a Lion alcolmeter™ (Lion laboratories Limited, Cardiff. UK).

2.3. Procedure

Given the high rate of weekend binge drinking in young adults testing was scheduled mid-week. Subjects were told to maintain their usual daytime and evening routines prior to participating in the study. In the 24 hours prior to testing subjects were asked to: abstain from engaging in strenuous physical activity; avoid any sudden disruption to their usual sleep/wake routine; avoid skipping meals and eating high calorie food outside of their usual meal times; and limit caffeine intake to no more than two cups per day. Alcohol was not permitted to be consumed for at least 48 hours prior to the day of testing. Lastly, prescription and

over the counter (e.g. pain and cough preparations) was not permitted to be used for at least 7 days prior to the study.

Participation in both alcohol trials was preceded by a six hour fast, which commenced at 1100 h Eastern Standard Summer Time (ESST), and testing began at approximately 1700h EST. Ketone bodies were assessed using urinalysis upon arrival in order to confirm that all individuals had complied with the fasting conditions. Alcohol was not permitted to be consumed for at least 48 hours prior to testing and all subjects were breathalyzed upon arrival to ensure that all recorded a BAL of zero.

Test Beverage: The test beverage consumed in both alcohol trials was Hardy's Regional Reserve Chardonnay 2005 white wine (McLaren Vale Vineyards, South Australia), containing 13% alcohol (315 KJ per unit).

Alcohol Dosage: The National Health and Medical Research Council of Australia define low risk alcohol consumption for males as no more than four standard drinks (40g alcohol) per occasion (NHMRC, 2001). In order to secure ethics approval to conduct the study the dosage of alcohol needed to adhere to these strict safety guidelines. Those who drank alcohol reported on average to consume 9.1 standard units (SD = 8.7 U) containing 10g alcohol on an average of 6.9 occasions per month (SD = 4.4 occasions). Therefore, it was deemed appropriate to set the dosage of alcohol for this study at the maximum amount of 40g alcohol.

Trial 1 (Serum + Salivary cortisol): Upon arrival subjects (n = 8), were asked to provide a 50ml urine sample for urinalysis prior to having 10ml of blood drawn from the left forearm for assessment of serum cortisol. Simultaneous saliva sampling for assessment of salivary cortisol was conducted while blood was withdrawn to ensure any stress effects resulting from the blood taking procedure were similar when taking the salivary and serum cortisol measures. Following the blood taking procedure participants were asked to slowly ingest a total of four standard units of alcohol (40g alcohol) over a 135-min period. Simultaneous blood and saliva sampling for the assessment of cortisol and measurement of BAL was performed a further three times at regular intervals while alcohol was being consumed. The rate of alcohol consumption was monitored to ensure subjects consumed alcohol at a similar rate during the study. As cortisol is released in response to physical or mental stress subjects were required to be seated at all times during the testing procedure. Physical activity or anything of a mentally stressful nature (e.g. movies, hand held video games) was not permitted at any time. During the study subjects engaged in quiet conversation with each other or played card games. At the completion of Stage 1 all participants reported moderate intoxication. No subject experienced gastrointestinal or other distress during the course of the study.

Trial 2 (Salivary cortisol): Upon arrival participants (n = 8) were required to provide a 5-ml saliva sample for measurement of cortisol and a 50-ml urine sample for urinalysis before consuming 40 g alcohol over a 135-min period. Saliva samples for the assessment of cortisol were taken; urinalysis was performed; and blood alcohol level (BAL) was assessed at regular intervals across a 135-min period. The rate of alcohol consumption was monitored to ensure subjects consumed alcohol at a similar rate during the study. Subjects were required to be seated during the testing procedure. Physical activity or anything of a mentally stressful

nature (e.g. movies, hand held video games) was again not permitted at any time during the study. Subjects engaged in quiet conversation with each other or played card games. All individuals reported moderate feelings of intoxication at the completion of the alcohol trial and no participant experienced gastrointestinal or other distress at any stage.

2.4. Statistical Analyses

Both the saliva (only) and serum+saliva trials employed a repeated measures design. The dependent variable was the level of cortisol (serum cortisol, salivary cortisol) and in both trials this was assessed across four time points (i.e. 0-min, 45-min, 90-min and 135-min). In order to achieve a moderate effect size $d = 1.0$, $\alpha = .05$, and Power = .75 using the sample size calculations listed in [36] a total of 21 participants would need to be recruited. However, with a repeated measures design the data from each participant is used at each measurement point so in effect eight participants in a repeated measures design with four measurement points is equivalent to data for 32 participants (i.e. $8 \times 4 = 32$), which is in excess of the 21 participants required. Thus, it was deemed statistically sufficient to recruit 8 participants for each trial.

Pearson's product-moment correlation analysis was used to assess whether a relationship exists between serum cortisol and salivary cortisol in the serum+saliva trial. A within subjects analysis of variance (ANOVA) was used to assess change in the level of the dependent variable (serum cortisol, salivary cortisol) when white wine is consumed across the four time points in the saliva (only) and serum+saliva trials. Any minor violation of sphericity was corrected using the Huynh-Feldt Epsilon correction. Results were classed as significant if the calculated probability was less than .05. All significant ANOVA findings were assessed post-hoc using paired samples t-tests.

3. Results

The average BAL recorded during the saliva + serum trial and the saliva (only) trials reached a mean peak of 0.08 mg/100 ml (SEM = .003 mg/100 ml) and 0.07 mg/100 ml (SEM = \pm .005 mg/100 ml), respectively, after four standard units of white wine (40g alcohol) had been consumed at 135-min.

The mean level of serum and salivary cortisol in the serum + saliva trial is graphically presented in Figure 1. No significant relationship was observed between the level of serum cortisol and salivary cortisol at any time point ($P > .05$). The ANOVA analysis also failed to reveal any significant differences in salivary cortisol across the 135-min alcohol consumption period ($F(3, 21) = 1.62, P = .22$). Inspection of the raw data showed a high degree of variability between subjects, which likely contributed to the non-significant finding. In contrast, a significant difference in serum cortisol concentration was observed across time points, ($F(3, 21) = 9.29, P < .01$). Post-hoc assessment confirmed that the average level of serum cortisol is significantly higher at 45-min when compared to 90-min and 135-min and at 90-min when compared to 135-min ($P < .05$).

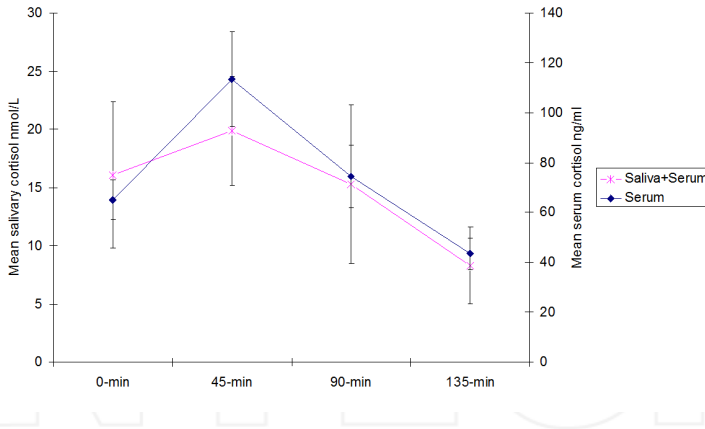


Figure 1. Mean salivary cortisol and serum cortisol obtained during the serum+saliva trial before (0-min) and after ingestion of white wine containing 40g alcohol (45-135 min). Data are shown as the mean ± SEM (n = 8).

The level of salivary cortisol in the saliva (only) trial during white wine consumption was noted to be significantly different across time points ($F(3, 21) = 5.46, P = .05$). Post-hoc assessment confirmed that the average level of salivary cortisol in the saliva (only) trial is significantly lower at 135-min when compared to 0-min, 45-min and 90-min and significantly lower at 45-min when compared to 90-min ($P < .05$). The mean salivary cortisol level measured during the saliva (only) trial is graphically compared with the serum cortisol data from the serum + saliva trial in Figure 2.

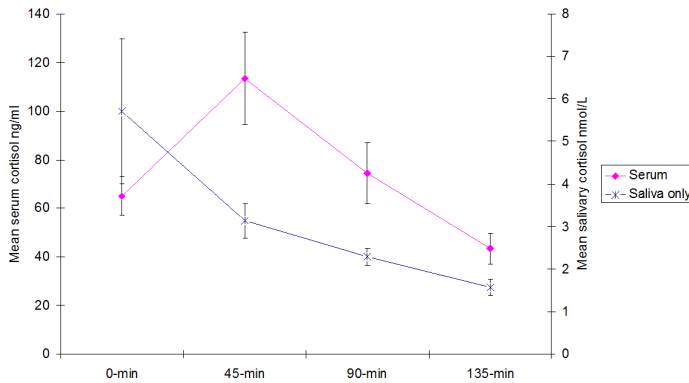


Figure 2. Mean salivary cortisol obtained during the saliva (only) trial (n = 8) and serum cortisol from the serum+saliva trial (n = 8) before (0-min) and after ingestion of white wine containing 40g alcohol (45-135 min). Data are shown as the mean ± SEM.

4. Discussion

The results of this study have shown that when alcohol is consumed under fasting conditions: the level of serum cortisol is significantly elevated almost immediately; the level of salivary cortisol when assessed during a blood taking procedure is not significantly altered over time; and the level of salivary cortisol when assessed in the absence of blood taking is significantly reduced over time.

Research has shown that blood alcohol concentrations exceeding 1 g/L can elevate plasma cortisol concentration [18]. However, other investigations using rodents and humans have indicated either no-change, or a significant decrease in plasma glucocorticoid levels following alcohol consumption [e.g. 2, 20-25]. Results of this study, which was conducted under fasting conditions are consistent with the suggestion that alcohol can promote an elevation in cortisol [18] and low to moderate alcohol intake has little [19], if any effect [20-24], on cortisol.

The collection of serum cortisol and salivary cortisol was conducted simultaneously and it could be argued that while the stress of the blood sampling procedure may have raised blood levels those in saliva may not have had time to equilibrate. However, it should be noted that the entry of cortisol from blood to CSF is a fairly rapid process [37].

In the present study there appeared to be little evidence for the suggestion that an initial alcohol-induced decrease in cortisol is later followed by a significant elevation in cortisol [25] either during or following alcohol consumption in serum or salivary cortisol measures. Our early work identified some food-induced recovery in the HPA axis following alcohol consumption [9], suggesting that the addition of nutritional factors can promote an elevation in cortisol. Moreover, we have also assessed the effect of beer, an alcoholic beverage that contains some carbohydrate, on steroid activity and we noted an elevation in salivary cortisol after 40 g alcohol [38]. Thus, differences in nutritional content of the alcoholic beverage consumed may be responsible for this discrepancy in cortisol findings highlighting the importance of nutritional factors when assessing the effects of alcohol on cortisol rhythmicity.

Researchers have previously claimed that differences in stress levels associated with the blood taking procedure [26] can falsely increase cortisol values. During the serum+saliva trial the level of serum cortisol was significantly elevated at 45-min while the level of salivary cortisol remained statistically unaltered. In contrast, the level of salivary cortisol in the saliva only trial is significantly reduced, which is consistent with previously published data obtained under similar experimental conditions [9, 38].

It has been suggested that as each individual may respond differently to the blood taking procedure the high degree of variability in stress response may be an important factor that could contribute to non-significant findings [27]. A large degree of inter-subject variability was noted in the salivary cortisol data during the serum+saliva trial, which most likely explains the non-significant result. The sample size in the present study, while statistically large enough, is relatively small given the high degree of variability observed. Therefore, the salivary cortisol data in the serum+saliva trial may need to be interpreted with caution.

An increase in the level of cortisol in CSF, in the absence of any elevation in plasma cortisol, has been reported previously [33]. In the serum+saliva trial there did not appear to be any significant relationship between serum cortisol and salivary cortisol, which suggests that the mechanisms underlying the release of cortisol in saliva and serum may not be the same. It has been suggested that the brain has the ability to synthesize steroid hormones such as dehydroepiandrosterone sulfate *de novo* [39]. However, whether this is also true for cortisol requires further investigation.

Cell membranes are highly permeable to alcohol and there is evidence that alcohol can increase membrane fluidity by expanding the volume and disordering the lipid components of the neuronal membrane [40]. The entry of alcohol into cells increases the concentration of solute and a hypernatremic dehydration condition may develop as cells gradually become saturated with alcohol. Both alcohol and cortisol can promote the release of K^+ from intracellular stores. However, the K^+ loss induced by alcohol is much larger [41] that is compensated by a Na^+ gain that more than doubles the glucose consumption by cells at higher alcohol concentrations [42, 43]. It has been suggested that the alcohol-induced increase in K^+ efflux is most likely due to extracellular K^+ directly antagonising the intoxicating effect of alcohol in the CNS [44]. The alcohol-induced increase in K^+ efflux increases glial depolarization [45] and glucose demand by neurons and astrocytes as additional glucose is required by astrocytes to take up the excess K^+ and store it [46]. An increase in K^+ efflux can stimulate GABA release [47], in order to protect against brain impairment caused by an increase in neuronal depolarization [48]. Alcohol's potentiation of GABA is specifically linked to the $GABA_A$ receptor gated chloride channel [49] and this may be due to Cl^- being needed by astrocytes in order to maintain electrical neutrality [46].

An increase in K^+ efflux could eventually develop into an extracellular acidosis [50], which in turn could promote a significant alteration in the activity of the HPA axis as a result of chronic osmotic stimulation [51]. A decrease in HPA axis activity under these conditions would be beneficial to the survival of the organism because cortisol decreases GABA activity at higher concentrations [52, 53], which could potentially increase the risk of brain injury due to an increase in neuronal depolarization [48]. Additionally, cortisol (similar to alcohol) can promote the loss of body potassium due to the release of K^+ from intracellular stores [41] and reduce glucose utilization and transport in neurons and astrocytes [54-56], which could increase the risk of hypoglycemia and hypoxia [57]. Thus, the significant decrease in salivary cortisol noted in the saliva (only) trial may have occurred due to an alcohol-induced alteration in HPA axis activity as outlined in the Salt and Water hypothesis [for review see 58].

5. Conclusions

The data here supports the suggestion that stress is an extraneous factor that can influence the cortisol data [26]. It was argued that consuming white wine under fasting conditions most likely promotes an alcohol-induced reduction in HPA activity [58]. The data in the present study has confirmed there is a need for strict methodological controls to be included

in any study assessing the effects of alcohol on the HPA axis. Cortisol is the body's major stress hormone. However, the role of cortisol in the human body is varied with cortisol also having a significant effect on energy regulation [10, 11]. Therefore, care should be taken when designing studies aimed at assessing the effect of alcohol on steroid hormones as any alteration in participant stress and/or nutritional status has the potential to increase inter-subject variability and potentially obscure the true effects of alcohol on the HPA axis [27].

Acknowledgements

I am grateful to Amy Lindner for assistance with subject recruitment, Bronwyn Stevens for her assistance before and during the experimental trials and Maureen Todkill and Janelle Perkins for their assistance with the blood sampling procedure.

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