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Physiology & Behavior 90 (2007) 43 – 53

PHYSIOLOGY & BEHAVIOR

Measuring cortisol in human psychobiological studies

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Received 3 October 2005; received in revised form 15 August 2006; accepted 28 August 2006

Abstract

The steroid cortisol is an extensively studied and important variable in developmental and other behavioral studies. Cortisol has been assayed by various methods using a range of substrates including blood, saliva, and urine. Cortisol in blood exists in two forms. While most is bound to carrier proteins, a small portion exists in a soluble free form. The informed choice of cortisol fraction and measurement method is critical for research. Such choices should be influenced by understanding the characteristics of the various cortisol fractions, along with their binding proteins' biological functions and relationship to the hypothalamic–pituitary–adrenal (HPA) axis. The goal of this paper is to familiarize researchers with key points for evaluating the choice of total and free cortisol in research as well reviewing various options for measuring free cortisol. These points are raised with special emphasis on their significance during pregnancy and the post-partum. Such information may prove useful in informing researcher's cortisol-related protocols and in the interpretation of cortisol data.

Keywords: Calculated free cortisol; Free Cortisol Equation (FCE); Cortisol-binding globulin (CBG); Free cortisol; Free cortisol index (FCI); Free hormone hypothesis; Perinatal; Pregnancy; Salivary cortisol; Venipuncture; Women

1. Introduction

Cortisol has long been used in human psychobiological studies as a biological marker of stress, anxiety and depression. Total and free cortisol have been measured both together [40,82] and separately (for review see [21]). Free plasma cortisol itself is usually not assayed directly but via a surrogate. One commonly used free plasma cortisol surrogate is salivary cortisol [56]. The correlative validity of salivary cortisol as a surrogate of free plasma cortisol has been widely discussed [58,59]. However the reasoning behind preferring free plasma cortisol to total cortisol is rarely addressed. Essex et al. [26] have justified the use of salivary cortisol by assuming that plasma free cortisol is the only biologically active fraction, an assumption based upon the Free Hormone Hypothesis. From its inception, the Free Hormone Hypothesis was controversial. It initially received considerable empirical support but recent research on the regulatory roles of corticosteroid-binding globulin (CBG) has added new doubts. This paper aims to present some of the basic assumptions and review relevant data, which may aid the researcher's choice for free cortisol. Apart from salivary cortisol, another, less-known surrogate for free cortisol is calculated free cortisol. This is presented as a viable alternative. While relevant to all human psychobiological research, this review will also discuss pregnancy and the postpartum, a period during which cortisol research is particularly problematic.

2. Cortisol

Cortisol is a glucocorticoid that affects every bodily system to such a large extent that it is difficult to characterize its actions succinctly [85]. One major function of glucocorticoids is the rapid mobilization of amino acids and fat from cells that make them available for use as energy as well as for synthesis into new compounds. This function plays a central role in organizing

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doi:10.1016/j.physbeh.2006.08.025

the body's response to physiological and psychological stressors. Glucocortocoids also have mineralocorticoid activity, as well as an anti-inflammatory effect upon traumatized tissues and they suppress the immune system. Centrally, glucocorticoids play an important role in maintaining many brain activities. Mizoguchi and colleagues [77] recently reported on the importance of glucocorticoids for prefrontal cortical cognitive function, specifically for working memory. In addition, glucocorticoids have multiple effects on human behavior such as sleep patterns, mood and the reception of sensory input. Cortisol is the most important of the human glucocorticoids and it is present in higher levels in women than in men [105].

2.1. Cortisol and the hypothalamic–pituitary–adrenal (HPA) axis

Cortisol is an end-product of the HPA axis. When stimulated, the hypothalamus secretes corticotrophin-releasing hormone (CRH). In response, the pituitary gland secretes adrenocorticotrophic hormone (ACTH), which in turn, stimulates the secretion of cortisol from the cortex of the adrenal gland. In general, the HPA axis self-regulates via negative feedback whereby elevated circulating levels of cortisol lead to suppression of CRH and ACTH release, thus reducing cortisol production. Cortisol in humans is secreted diurnally, in response to pulsatile trophic hormone stimulation [16,57,119] with cortisol levels peaking early, prior to awakening, and decreasing progressively during the day to reach low levels in the evening. The pulses of CRH and ACTH vary in amplitude throughout the day, with the amplitude decreasing during the diurnal trough. Calculations indicate that the 'pulsatile' and 'circadian' components are separate secretory modes that can be independently regulated.

Both stress [50] and the circadian cycle [25] are intimately associated with HPA axis activity, although the central pathways by which they are linked to the hypothalamus are incompletely understood. Cortisol circulates in the blood in both free and bound forms; cortisol's biological half-life is around 80 min. In plasma, cortisol is predominantly bound to corticosteroid-binding globulin, with a small amount bound loosely to albumin, and the remainder free. The remaining free cortisol molecule is lipophilic and has a low molecular weight (MW ~362 Da), passing from capillaries into tissues mainly by passive diffusion.

2.2. Corticosteroid-binding globulin

Corticosteroid-binding globulin (CBG) is a glycoprotein with a molecular weight ranging between 50 and 60 kDa. This variation in molecular weight is due in part to genetic variants of CBG [24] and the increasing molecular weight during pregnancy due to differential glycosylation [76]. CBG binds cortisol stoichiometrically and with high affinity [2]. The physicochemical and binding properties of the various plasma proteins for cortisol have been reviewed elsewhere [117]. Transcortin is a synonym for CBG, implying a transport function. However CBG is not essential for cortisol transport as this role can be accomplished by albumin, and further, cortisol itself is sufficiently water soluble at physiological concentrations. Haourigui et al. [48] claim that dietary free fatty acids (FFA) may have an impact on bioavailability of glucocorticoids by inducing conformational changes in CBG mostly mediated by monounsaturated FFA, especially C18:1.

3. The Free Hormone Hypothesis

It is commonly and perhaps uncritically assumed that free cortisol and only free cortisol is the biologically active fraction. This assumption finds its fullest expression in the Free Hormone Hypothesis. This hypothesis [72] predicts that the biological activity of a given steroid correlates with the free protein-unbound concentration rather than with the total concentration. The physiological background to this hypothesis is that nonpolar steroid hormones, having very low solubility in aqueous extracellular fluid, circulate in the bloodstream largely bound to specific high-affinity, low-capacity circulating carrier proteins, as well as binding to lower affinity high-capacity non-specific proteins such as albumin [66]. According to the hypothesis, only free cortisol is available for movement out of capillaries and into cells. The major advantage of this hypothesis is that it provides a generalized theoretical framework for steroid actions. However, limitations of the hypothesis relate to the fact that it does not apply uniformly to all steroids (e.g., it fits estrogens less) and it does not take into account any additional biological roles of the steroid binding proteins. Analysis of the effects of intra-capillary proteins' binding reactions on target-tissue hormone uptake is also complex [23] and the hypothesis may not be valid for all hormones with respect to all tissues [72]. Notwithstanding this, the hypothesis was thought to account for most cortisol action and ovarian follicular biology [2]. Indeed the broad correlation between endocrine status and free hormone levels in serum formed the basis for the hypothesis [23]. The main tenet of the hypothesis is the exclusive biological activity of the free hormone, but free hormones are also equally free to undergo hepatic or other clearance. Since the balance of these two effects is unpredictable, there is no theoretical basis to believe that free hormone measurement represents a more biologically active moiety of a circulating hormone [66].

A corollary of the free hormone hypothesis is that the protein-bound concentration is physiologically irrelevant [23]. However Tait and Burstein [108] claim that hormones that are only loosely bound to albumin should be considered free as illustrated by cortisol which binds to albumin with weak affinity. In circulation there is 800–1000 molar excess of albumin over CBG and its affinity for cortisol is 1300 times weaker [84] and approximately twice as much cortisol is 'bound' to albumin than is free, 6–20% versus 3–10%, respectively [2,15]. If Tait and Burstein [108] are correct in their assumption, then that would in effect mean that there is more 'free' cortisol in ostensibly bound cortisol than is truly free.

Paradoxically the hepatic uptake of free cortisol, the amount of cortisol catabolized by the liver at any one time, is three times greater than the amount of free cortisol in plasma. This problem

can be resolved by the assumption that the dissociation rate of cortisol from binding proteins is very rapid, thus replenishing expended free cortisol pools [84], further clouding the distinction between free and bound cortisol. As previously mentioned, the physiological role of CBG is diverse. It plays a role in the regulation of cortisol availability to target tissues [8] and like bound thyroxine [71], it may offer a more uniform delivery of hormone to tissues. In addition, there is direct evidence for the cellular uptake of the corticosterone–CBG complex, suggesting that this complex plays a role in steroid hormone action complex [100].

A more subtle steroid delivery mechanism for CBG is a consequence of it being a member of the SERPIN superfamily whereby cleavage by human neutrophil elastase, at sites of inflammation, results in a marked drop in its affinity for cortisol, thereby delivering steroid to that site [90]. It is also becoming clear that CBG may also bind directly to membrane receptors, offering another alternative delivery mechanism of steroid to target cells [104] where membrane recognition may be glycoform dependent. More acidic glycoforms appear in maternal plasma in late gestation [76] and pregnancy-specific CBG has been shown to have a higher affinity for syncytiotrophoblast cell membranes [103]. Moreover CBG-bound cortisol has been shown to be internalized in MCF-7 cells via a CBG membrane receptor resulting in the accumulation of cAMP [95]. More recently, the uncovering of endocytic pathways for the cell-type-specific uptake of protein-bound steroids [46] further challenges the exclusiveness of the free hormone hypothesis and suggests that the two hypotheses should not be regarded as mutually exclusive but rather complementary in their ability to explain the diverse functions of CBG.

Since its inception, the free hormone hypothesis has been contentious and it remains so, constituting at best an approximation [23,66]. This would seem to raise serious doubts about the axiom of free cortisol being the only fraction involved in biological activity, of whatever kind.

4. Cortisol: brain, behavior and development

In addition to passive diffusion, recent studies indicate that cortisol transport across the blood–brain barrier may be a regulated process [70]. Access of cortisol to the brain has been shown to be limited by such factors as the multidrug-resistant P-glycoprotein (Pgp). Pgp is a transmembrane protein that actively transports a broad range of substrates from the intracellular compartment to the extracellular space. Pgp is expressed in the capillary endothelial cells of the blood–brain barrier, and controls the access of many compounds to the brain [96]. Antidepressants may inhibit these steroid transporters increasing cortisol access to the brain. This results in increasing glucocorticoid receptor (GR) expression and function [87] and enhanced glucocorticoid-mediated negative feedback on the HPA axis. Levels of P-glycoproteins are also regulated in the placenta during the course of pregnancy. Taken together, these findings call for a careful reevaluation of many of our assumptions about the mechanisms and function of corticosteroid action, which is regulated through a very complex process.

Cortisol has been shown to impact on maternal behavior and such effects have been studied in birds [67], baboons [5] and in humans [31]. The relationship between cortisol and distress has become of major interest to researchers in social and emotional regulation, particularly in the field of child development, reflecting a growing awareness of the possible links between maternal hormones and fetal/infant development. In human mothers it was concluded that cortisol may play an important role in the initiation or early expression of maternal behavior. Others have hinted at the potential negative impact of maternal distress during pregnancy on fetal growth and development via hormonal substrates such as cortisol. There is interest in the direct relationship between maternal cortisol levels during pregnancy and their ability to predict newborn cortisol levels [30] although others have shown that fetal HPA axis response to stress is independent of the maternal response [35]. Since maternal cortisol is known to cross the placenta, there is concern that exceptionally high cortisol levels, such as may be observed among depressed mothers, may affect neonatal biochemical profiles [22]. The relationship between antenatal maternal depression and anxiety has also been described [22,53] as well as the links between postnatal maternal mood and offspring cortisol levels [4,45] although the link between cortisol and either prenatal or postpartum depression is tenuous. Nestler et al. [81] reviewed research with the purpose of generating a neurobiological explanation of depression. They suggest that glucocorticoids play a major role in numerous theories, particularly since their interactions with the limbic system and the HPA axis provide insights into the links between stress and depression. Others advocate a non-linear, more differentiated approach [11] to the relationship between stress and cortisol. Levels of other cortisol transport controllers, besides CBG, such as P-glycoproteins are regulated in the placenta during the course of pregnancy. A two-fold decrease in the mean expression of P-glycoprotein between early and late gestation human placental samples has been reported [33], suggesting that the placenta's ability to protect the fetus from cortisol and xenobiotic exposure is greater in early pregnancy than at term [32].

As with HPA axis activity, researchers need to be aware of what is meant by the biological activity of cortisol. Is cortisol a biological marker for a given biological activity [52] and if so, which form of cortisol might best be used to index HPA activity. Ultimately, biological activity should be assayed using a bioassay. However, the characteristics of bioassays make them unattractive for psychobiological studies.

5. Cortisol in medical and psychobiological research

Clinical interest in the measurement of cortisol is due to the fact that disturbances of cortisol levels are evident in many pathological states. Extreme hypercortisolism leads to the development of Cushing's disease whereas extreme hypocortisolism leads to Addison's disease [43]. About half of the patients with either spontaneous or iatrogenic Cushing's syndrome also show a degree of psychological disturbance [101]. Hypercortisolism has been similarly associated with

major depression [112,74] and it has been suggested that as many as 60% of the cases of major depression are associated with hypercortisolism [91]. Adrenal insufficiency may similarly result in psychiatric disturbances [80]. Congenital adrenal hyperplasia (CAH) also requires careful monitoring to ensure adequate cortisol replacement [83]. Patients suffering from extreme physical trauma as well as patients who have undergone major surgery also require close monitoring of cortisol levels as compromised adrenal function is a common complication of surgery. Dysregulation of the HPA axis resulting in hypercortisolism has been proposed as a mechanism by which depression may evolve from chronic stress [107]. Hypercortisolism in severe depression expresses itself in a shortened nocturnal quiescent period, an earlier morning rise, and an overall increase in cortisol levels. Conversely, there is increasing evidence that hypocortisolism is present in stress-related disorders and chronic fatigue syndrome (for review see [49]). Recently, it has been proposed that at least five interacting hypothalamic peptidergic systems, one of which is the HPA axis, are involved in depression [105]. Yet, the neurobiology of the relationship between cortisol and psychiatric conditions of stress and depression is still not well understood.

6. Cortisol and CBG in health and disease

Studies show that CBG levels may differ in response to factors such as food availability and exercise. Plasma CBG decreased in white crowned sparrows following fasting [68] and in rats, exercise-induced stress reduced circulating CBG [78]. CBG may also be involved in cortisol-driven obesity in animals [85,39] and humans [115] and appears to be associated with insulin secretion [29] although its role as a marker of insulin resistance in obese males is less certain [64]. More recently, using age-matched males, lower levels of CBG in obesity were reflected by higher levels of circulating free cortisol, potentially offering a more favorable environment for adipogenesis [63]. Taken together, these findings suggest that CBG levels and binding capacity may be important determinants in the energy-regulation process.

6.1. Pregnancy

Fluctuations in CBG typically occur during pregnancy [73, 92] affecting free cortisol levels, leading to a change in the relationship between total and free cortisol levels. This fact makes pregnancy studies a particularly relevant context for this discussion. CBG levels become elevated during pregnancy [1] and rise between weeks 10 and 20 of gestation [27]. The reason for the rise of CBG is currently unclear, although originally it was proposed that rising estrogen levels were responsible [102]. During pregnancy the increases in immunoreactive CBG and total cortisol are grossly parallel but the ratio of CBG available for cortisol occupancy declines over term, likely the result of increasing levels of progesterone and 17-OH progesterone, which also has high affinity for CBG [20]. The net result is mild circulating hypercortisolism in late pregnancy, which might simply be an adjustment to the set point of the HPA axis [98]. In addition, the steroid microenvironment at the fetomaternal interface is vastly different from in the periphery, presenting lower affinity constants of CBG for cortisol compared to plasma, thus biasing CBG loading towards progesterone [9]. This offers a potential shuttle for moving progesterone from the maternal intervillous circulation to placental target tissue. For these reasons, the limitations of measuring not only total but also free cortisol (whether by blood or saliva) over pregnancy need consideration.

7. Cortisol testing

How cortisol is measured depends to a large degree upon which form of cortisol is being assayed. As recently as the late 1990s, total cortisol was the usual form assayed in most studies and was implied rather than stated. This lack of specification makes interpretation of results difficult and may have contributed to the lack of clear distinction between the various forms of cortisol, their characteristics, and their implications. Bound cortisol is cortisol bound to its various binding proteins: CBG, SHBG (sex-hormone-binding globulin) and albumin. Free, or unbound, serum/plasma cortisol is the fraction of cortisol that is not bound to binding proteins. Total cortisol is the sum of the free and the bound fractions. Salivary cortisol is free cortisol that has entered into the saliva glands primarily by passive diffusion. Urinary cortisol excretion results from glomerular filtration and is a useful index of integrated 24-h plasma free cortisol. Mean urinary cortisol values are 130±104 nmol/24 h with a range of 47–417 nmol/24 h [79].

Total cortisol is usually measured by immunoassay either by in-house methods or by readily available commercial methods. Immunoassays for cortisol may cross-react with other steroids potentially leading to variability in measured levels. Methods for assaying free cortisol are complex, time-consuming, and expensive and include ultrafiltration, equilibrium dialysis, and steady-state gel filtration. No kit for simply assaying free serum/plasma cortisol is presently available, which may preclude a wider use of directly measured free cortisol in studies with large numbers of subjects.

Free cortisol is usually derived by calculation from measurements of total cortisol and plasma CBG binding capacity [6] or total cortisol and plasma CBG [15]. The latter Coolens calculation is commonly used in humans and takes into account the ratio of albumin-bound to unbound cortisol assuming normal plasma albumin. It will be called for the purposes of this review the Free Cortisol Equation (FCE) and is shown below.

$$U^{2*}K(1+N)+U[1+N+K(G-T)]-T=0$$

where U, T and G represent the molar concentrations of free cortisol, total cortisol and CBG, respectively, in μM. K is the affinity of CBG for cortisol at 37 °C and N is the ratio of albumin-bound cortisol to free cortisol. Assuming a K value of 3×10^{-7} M^{-1} and a value for N of 1.74, U and Z were expressed as follows:

$$U=\sqrt{Z^2+0.0122C}-Z^{\mu M}$$

$$\text{wherein } Z=0.0167+0.182(G-T)\ \mu M$$

A normalizing index, the free cortisol index (FCI), was described which is simply the ratio between measured total cortisol and CBG measured by immunoassay [64] which correlates well with free cortisol measured by steady-state gel filtration ($r^2=0.81$).

Salivary cortisol has been assayed with the same kits as used for assaying total cortisol in serum, with adjustments for sensitivity to cope with the much lower levels of cortisol in saliva. Furthermore, salivary cortisol levels are only 50–70% of serum free cortisol levels [73] due to the conversion of cortisol to cortisone by 11β-hydroxysteroid dehydrogenase type 2 activity in saliva. Differences in saliva cortisol levels determined by either radioimmunoassay or enzyme immunoassay emphasize the importance of methodology when comparing results from different studies [93]. Differing antibody characteristics and possibly standardization are the likely cause and show the importance of deriving method-specific reference intervals. This is further illustrated in the screening test for Cushing's disease, where midnight saliva cortisol cut-off values are more than double, depending on the method [86,113].

Urinary cortisol is also generally measured by immunoassays. The necessary additional sensitivity has been achieved by using high-affinity antisera that react specifically with the D ring of cortisol. Urinary cortisol has been evaluated both in developmental [22,29] and affective research [69] and its clinical relevance has been demonstrated in some assays [65]. However, reported true mean values vary widely and their levels have become increasingly discrepant [89], perhaps partly due to methods which may not distinguish between cortisol and its metabolites and which may be compounded by larger urine volumes [28]. Urinary cortisol will not be discussed further in this paper.

8. Considerations for sampling cortisol and CBG from blood and cortisol from saliva

8.1. Sampling cortisol from blood

Each procedure and methodology has well recognized advantages and disadvantages. Sampling cortisol in blood may have insurmountable drawbacks for researchers: it requires medical staff and specialized equipment, is costly and it may be considered invasive by some populations. Although cortisol is a stable molecule at room temperature, plasma may require special handling as it could be considered a biohazard.

Venipuncture has also been considered a drawback of blood sampling, especially where evaluations are not based on repeated sampling. The assumption has been that venipuncture elicits a cortisol response that could lead to artificially raised cortisol levels. While this might appear logical, it does not seem to be based on specific findings. There are no data to suggest that venipuncture may be a preanalytical variable that affects the laboratory testing of cortisol in any of its forms [118]. A corticosterone stress reaction in rats took 5 min to initiation of a rapid spike followed by a continuous rise [34]. Initiation of a cortisol stress response in humans takes approximately 20 min following hand immersion in iced water [56] with pregnant women being the notable exception. Deinzer et al. [19] in a study on parachutists, found cortisol responses 20 min after jumping. Interestingly, Gitau et al. [35] found no change in maternal cortisol levels 10 and 20 min after either fetal blood sampling or intrauterine transfusion, both performed without sedation. However, as gestation ranged from 22 to 35 weeks, some of these mothers may have been hyporeactive, since the HPA axis becomes hypofunctional towards the end of pregnancy [97]. Many developmental studies report cortisol results from a single venipuncture [7,12] although the response may occur long after stimulation. There appears to be some confusion between the instantaneous norepinephrine reaction to immediate threat (fight, flight and freeze) and the slower cortisol stress response. We would therefore conclude that if other considerations indicate the use of blood for cortisol sampling it should not be automatically excluded, although whether basal conditions persist at the time of sampling will be difficult to determine.

The 'white-coat' effect is also a consideration and it is strictly defined as clinic blood pressure minus ambulatory blood pressure. This has been associated with HPA hypersensitivity to stressors [106] although this finding is based on plasma cortisol sampling 30, 60, and 120 min after the challenge. Accordingly, the 'white-coat' effect cannot be disregarded if testing is carried out where both the testing situation and personnel are unfamiliar, and in situations where subjects remain in anticipation of venipuncture for an extended period of time.

Different timing methodologies for sampling cortisol are employed depending upon clinical or research objectives. Single measures are used to explore associations between cortisol and physiological or affective state characteristics. Multiple within-subject measures may be used to first establish baseline levels and then to note the development over time of a response to a stressful stimulus [56]. Diurnal measurement, a special subset of multiple measures, is designed to evaluate the integrity of the diurnal cycle. Cortisol levels are expected to be relatively higher in the waking hours and lower in the evening. The diurnal cortisol cycle, while slightly altered, is maintained during pregnancy despite the changes in HPA axis [73]. Women suffering from anorexia nervosa and Cushing's syndrome patients show increased cortisol secretion via amplified pulsatile trophic hormone [75,111]. The collection of cortisol over a 24-h period can be used to examine these trait characteristics. Since cortisol secretion is episodic, the 24-h mean-concentration can be used to establish whether cortisol levels are pathologically high or low, as they are in various disease states.

In the clinical setting, total plasma cortisol is often used initially for establishing whether a normal diurnal pattern of cortisol exists. Lack of diurnal variation could indicate the need for further testing. A random timed cortisol can provisionally evaluate whether the level is within the reference range. A plasma cortisol less than 80 nM at 0900 h provides presumptive evidence of adrenal insufficiency while levels greater than 300 nM likely exclude adrenal insufficiency [44]. However, total cortisol is not always diagnostically useful and many consider that free cortisol provides a better test of adrenal function than total cortisol. Hamrahian et al. [47] found that

critically ill patients with hypoproteinemia (albumin ≤ 2.5 g/dL) had subnormal serum total cortisol although their adrenal function was normal. As said, while the routine assay of plasma free cortisol is not readily available, the FCI provides a simpler option. Le Roux et al. [60] used the FCI to evaluate HPA axis function in severely ill patients undergoing surgery and Vogeser et al. [114] used calculated free cortisol to evaluate HPA axis function in patients following cardiac surgery. Both groups found that total cortisol does not appropriately reflect activation of the adrenal cortex compared to either FCI or calculated free cortisol. The FCI has also been used to study congenital adrenal hyperplasia [13] to follow the time course of patients suffering septic shock and trauma [8]. Measuring free cortisol in clinical settings is likely to become routine [54] particularly in septic shock and sepsis where both FCE and measured free cortisol are likely better guides cortisolemia since they correspond more closely with severity of illness than total cortisols [53]. Current consensus is that measured free cortisol is the ideal approach, with calculated methods a reasonable alternative for assessing HPA axis function. An alternative approach using other ACTH-dependent steroids such as cortisol:DHEA(S) has recently emerged [30].

There is considerable variability in reported reference range intervals not only for total plasma cortisol but also for free cortisol and CBG. Normally free cortisol is less than 6% of total cortisol [2] but as the total cortisol exceeds saturation of CBG the percentage of free cortisol increases [10]. In the extreme case of CBG deficiency, the free cortisol fraction is maximal, around 30%, since remaining cortisol is sequestered by serum albumin which is in vast molar excess [62]. The percentage of free cortisol within an individual can fluctuate due to both endogenous and exogenous factors, which may include illness, stress and trauma.

When sampling cortisol in blood there is a need to consider any transient or longer-term changes in CBG, which binds the majority of cortisol, or albumin, which can bind up to 20% of circulating cortisol [15]. Whereas congenital or other anomalies of CBG and albumin are rare enough not to be of concern for researchers studying normal populations, researchers studying pregnant women may wish to understand the possible effects of CBG on circulating cortisol levels.

There are a few reports of individuals having CBG variants with low cortisol-binding affinity [24] and even a case of complete congenital lack of CBG [109], a condition which was until recently unknown but predicted to be lethal [99]. This patient and another with low normal CBG levels showed deficient total cortisol responses following HPA challenge but were appropriately normal when free cortisol levels were considered [17,62].

8.2. Sampling cortisol from saliva

Measuring cortisol in saliva has many advantages including the ease of sampling [59]. It is stress-free, non-invasive, and allows for frequent and rapid sampling. Trained staff and specialized equipment are not necessary and sampling can take place outside of a laboratory allowing for sampling at home and at several times throughout each day. Cortisol in saliva is stable at room temperature and the costs of handling and processing are greatly reduced. However salivary cortisol is not without disadvantages [116]. Home testing often suffers from major problems with compliance [41] and subjects may provide insufficient saliva [55] or deviate from instructions [116]. Saliva provided after eating or drinking substances with low pH (i.e. fruit juices) [36] as well as the presence of blood in saliva due to oral lesions [4] may artificially raise cortisol levels. Some disadvantages may be resolved by sound planning, rigorous follow-up and other strategies. Blood-contamination can be determined by assaying for the presence of transferrin especially in saliva samples where the cortisol exceeds 2 μg/dL (55 nmol/L) [4]. Collecting saliva in a clinical setting can overcome some disadvantages.

Salivary cortisol has been used as a tool for physiological and diagnostic studies. These include circadian studies in term and preterm infants [11], as a predictor of Cushing's syndrome in children [110], and for following the cortisol response to exercise-induced stress and CRH stimulation [38]. It has also been used successfully for monitoring adrenal function of outpatients using topical intranasal glucocorticoids for rhiniosinusitis [88].

Since the early 1980s salivary cortisol has been used in endocrinology, psychobiology, and behavioral medicine research studies [59] with increasing popularity, likely the result of the attractiveness of non-invasive sampling. Prenatal maternal cortisol levels correlate with neonatal crying, fussing and negative facial expressions [18] and salivary cortisol levels in children appear linked to maternal stress in infancy [26] although others found no correlation between salivary cortisol in nulliparous women during pregnancy and temperament in toddlers [42]. Unlike salivary cortisol, calculated free cortisol is rarely used in developmental studies. Research from our laboratory has recently analyzed cortisol levels, using the FCE method [15] during pregnancy and the early post-partum in a non-clinical sample of women [61].

9. Free cortisol

There are many considerations that may lead the researcher to make an informed decision to use free cortisol instead of total or bound. In that case, a decision of how to measure free cortisol is required. Currently no kit is available for the direct measurement of free cortisol in serum or plasma. Free cortisol is usually measured "in house" for comparative and other research purposes often with small numbers of subjects as it is technically complex and costly. Currently it is not really suitable or practical for larger studies although alternatives exist. Amongst them are salivary cortisol, FCE and FCI. The latter two require blood sampling.

Salivary cortisol is a reliable reflection of (total) plasma values [59] and circulating free cortisol with correlation coefficients between cortisol in saliva and cortisol in serum ranging between $r=0.71$ and $r=0.96$, thus determining proportions of total variance between $R^2=0.054$ and $R^2=0.864$. Goodyer et al [37] reported that salivary cortisol levels correlate highly with serum levels ($r=0.6$–0.9 or proportions of total variance ranging between $R^2=0.36$ and $R^2=0.81$) and others claim that salivary cortisol is an excellent substitute for free plasma cortisol [73,88]. Despite the reported correlations between salivary cortisol and

unbound cortisol, important diurnal differences between the two measures have been noted [73] and there appears to be discordance at higher concentrations and regarding the variability of repeated measures, which is more pronounced with saliva cortisol [94]. Furthermore the correlation between salivary cortisol and plasma free cortisol appears to be subject-specific as considerable variability is found between subjects for daily-paired samples (Fig. 1).

Perhaps one of the most common and non-empirical bases for the comparison of saliva cortisol to free serum cortisol has been the assumption that, like free cortisol, saliva cortisol is unbound. However Chu and Ekins [14] found that 14±4% of cortisol in saliva is bound. These and other findings suggest only that the distinction between a correlation and actual biological identity be judicially maintained.

Saliva cortisol has been used extensively in psychobiological research and is less common in medical research where both FCE and FCI are used. This could be partly due to the difficulty of obtaining blood in a psychobiology setting compared to a medical setting. While the FCE, like salivary assays, does not directly measure free cortisol, it provides a free cortisol correlate based on measurements in the same biological fluid. Recently we compared salivary cortisol as measured by ELISA with free cortisol as estimated by a calculated free cortisol method (FCE), as assessed by a free cortisol index (FCI) and as measured by ligand binding/ultrafiltration which, like equilibrium dialysis, can be considered a "gold standard". We also compared calculated free cortisol (FCE) with free cortisol as measured by ligand binding/ultrafiltration. The results of this study, $N=246$, showed that measured plasma free cortisol values corresponded more highly with the FCE ($R^2=0.754$) than with salivary cortisol ($R^2=0.501$). Further, only low degrees of common variance were found between salivary cortisol and both the FCE ($R^2=0.289$) and the FCI ($R^2=0.217$). We note that these findings are within-subject data taken from a relatively large cohort. Taken together, these are not surprising results. However, from a practical standpoint they do suggest that FCE could be a preferred method for use in research, although this conclusion is based on a single but large study.

Table 1
Examples of calculated free cortisol levels as calculated from total cortisol and corticosteroid-binding globulin

Total cortisol (nmol/L)	Corticosteroid-binding globulin (nmol/L)	Calculated free cortisol (nmol/L) (Coolens et al. [15])
331	408	40
477	358	81
490	352	86
582	1144	27
662	1397	25

To calculate free cortisol levels, the research entails all of the drawbacks which blood sampling and processing bring. Calculated free cortisol is additionally cumbersome because calculation requires that both total cortisol and CBG be first separately assayed on the same sample. However, this does not require a large amount of blood, five milliliters of blood being more than sufficient even if divided into two aliquots. Commercial kits are available for each assay, but careful planning is needed since the shelf life of the CBG kits may be limited. The total cortisol and CBG values are then used in the equation to calculate free cortisol. Data from one of our own studies using calculated FCE demonstrates the non-linear relationship between total cortisol and calculated free cortisol (see Table 1).

10. Summary and conclusions

Cortisol and its fractions continue to be of interest to researchers in the area of psychobiology. They have been variously assayed in medical and developmental research.

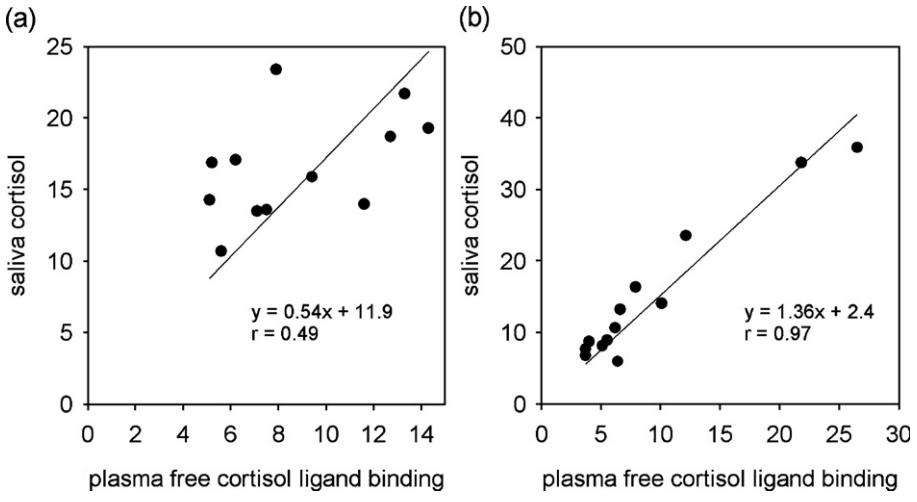

Fig. 1. Correlation of saliva cortisol (nmol/L) and measured plasma free cortisol (nmol/L) in paired samples from two normal individuals (a and b). Saliva cortisol was measured by ELISA and plasma free cortisol by ligand binding/ultrafiltration.

Initial interest in total cortisol has largely been replaced by interest in free cortisol, which may be related, in part, to the free hormone hypothesis. However this hypothesis has limitations. The exclusivity of free cortisol as the only biologically active fraction needs reevaluating. Similar questions may arise when considering the primacy of free cortisol as a reflection of HPA axis function, although serum free cortisol measurement is probably the most reliable method to assess HPA axis function in critically ill, hypoproteinemic patients [3,55].

Research measuring free cortisol using saliva cortisol as a surrogate requires some caution as several non-trivial difficulties may exist. Although saliva has advantages, the issues of compliance, variability, and identity between salivary and free cortisol are drawbacks. Calculated free cortisol, a lesser-known free cortisol surrogate, although more invasive, complex and costly than salivary cortisol, is closest to the gold standard of plasma free cortisol measured by either equilibrium dialysis or ultrafiltration [51]. Despite this, as a practical option, salivary cortisol is still a useful alternative.

We hope that this paper has helpfully touched upon relevant questions concerning cortisol, its fractions and surrogates, and their research significance. We tried to provide methodological and theoretical information regarding choice of cortisol fraction and cortisol measurement in the context of psychobiological research. We have considered some of the criteria for choosing between use of total cortisol and free cortisol. If free cortisol is deemed appropriate, then consideration is required as to its diverse surrogates, whether salivary cortisol, calculated free cortisol or the free cortisol index. The future development of simple kits for directly measuring plasma or serum free cortisol will help obviate the choice of surrogate markers. For cortisol to be an important factor in psychobiological research, care must be taken in understanding the biological significance of cortisol and it fractions. Planning, interpretation and comparison of cortisol data can only be carried out based on understanding the forms of cortisol and options for its measurement.

Acknowledgements

This paper was written as part of a Ph.D. dissertation by the first author, in the Interdisciplinary Program in the Brain Sciences, Bar-Ilan University, Ramat-Gan, Israel.

Orna Zagoory-Sharon was partially supported by the Gonda Brain Research Center and by the US–Israel Bi-National Science Foundation. Research in Aron Weller's laboratory is partially supported by the Israel Science Foundation (02/771), by the US–Israel Bi-National Science Foundation (2001-198), and by the Paula Rich Center, Bar-Ilan University. Research in Ruth Feldman's laboratory is partially supported by the Israel Science Foundation (01/945), by the US–Israel Bi-National Science Foundation, and by the March of Dimes Foundation (#12-FY04-50). The authors thank an anonymous reviewer for extensive advice and many important references and Ilanit Gordon for her technical assistance in the revision of the manuscript.

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