Cortisol cut-points for the glucagon stimulation test in the evaluation of hypothalamic pituitary adrenal axis

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Abstract. Diagnosis of adrenal insufficiency requires evaluation by dynamic stimulation tests. The insulin tolerance test (ITT) is accepted as the gold-standard test for the evaluation of hypothalamo-pituitary-adrenal (HPA) axis but the test is unpleasant and dangerous. Although it takes more time, glucagon stimulation test (GST) is a good alternative to ITT. The primary aim of this study was to compare the ITT and GSTs in the evaluation of HPA axe in patients with pituitary disorders. We conducted a prospective study in which ITT and GST were performed within 7 days in 81 patients. Serum cortisol was measured. We divided our population in Group 1 (G1): Adrenal Insufficiency (Peak cortisol under ITT <200 ng/mL) and Group 2 (G2): normal response (Peak cortisol under ITT >200 ng/mL). Receiver-operating characteristic (ROC) analysis was performed to identify the thresholds for GST. The mean peak of cortisol under GST was not significantly different from that obtained after ITT in the whole cohort (182.67 ± 89.07 ng/mL vs. 179.75 ± 79.01 ng/mL), and it was significantly reduced in patients of G1 (p < 10−3). ROC curve analysis showed that the best diagnostic accuracy was obtained with a peak cortisol cut-off to GST of 167 ng/mL (sensitivity, 89%; specificity, 79%). Using this cut-off, 86.4% of the patients were correctly classified. In our prospective series, GST is a potential accurate and safe alternative test for the assessment HPA. Test-specific cut-offs should be applied to avoid misinterpretation.

Key words: Glucagon stimulation test, Insulin tolerance test, Hypothalamic pituitary adrenal insufficiency

ADRENAL INSUFFICIENCY is a vital but rare condition. Its prevalence is estimated at 150 and 280 cases per million inhabitants [1]. It results from a dysfunction affecting one or more levels of the hypothalamic-pituitary-adrenal (HPA) axis, leading to primary insufficiency when the damage affects the adrenal glands giving Addison’s disease or secondary in case of hypothalamo-pituitary damage. Accurate diagnosis of secondary adrenal insufficiency is crucial in patients as the decision for glucocorticoid replacement is based upon the diagnosis of HPA axis insufficiency. The use of stimulation tests is necessary for early diagnosis because of the insufficiency of static cortisol and ACTH in the diagnosis process. If in Addison’s disease a low-dose (1 μg) ACTH stimulation test was suggested to be the best test [2], the investigation of corticotropic insufficiency involves several methods with varying limits depending on the feasibility, risks and their adverse effects [3].

The insulin tolerance test ITT is a cumbersome test during which medical supervision is required although it is accepted as the gold-standard test in the evaluation of HPA and GH axis in patients with pituitary disorders [3]. Furthermore, it is contraindicated in patients with epilepsy, cerebrovascular or cardiovascular disorders, and may present side effects such as seizures [4]. Some alternative tests were proposed instead of ITT such as the ACTH stimulation test or glucagon stimulation test (GST). The (GST) appears to have a better diagnostic utility compared to the Synacthen test in comparison with the ITT [5]. Proposed for the first time in 1974 by
Rao & Spathis [6], the GST allows the evaluation of the somatotropic axis such as insulin hypoglycemia, but with fewer and less adverse effects [7]. The GST like the ITT stimulates both the ACTH and GH secretion, and it was suggested as an alternative stimulation test in terms of efficacy [8].

The present study was designed to evaluate the diagnostic ability of the glucagon stimulation test in the assessment of adrenal function in patients with pituitary disorders. The ITT was regarded as the gold standard test for the diagnosis of central adrenal insufficiency.

Patients and Methods

Patients

Eighty one patients (44 men, 37 women) with pituitary disorder were included in the study after oral and informed consent, from January 2016 to June 2017 in the Department of Endocrinology of the University Hospital of Farhat Hached Sousse. The mean age of the patients was 35.83 ± 19.62 years. Exclusion criteria were: major systemic diseases, contraindications to ITT such as epilepsy, cerebrovascular or cardiovascular disorders, Cushing’s disease, known diabetes mellitus who required medication and estrogen replacement. Long-term corticotherapy patients made a correct decreasing in order to stop steroids. They were under prednisone with a mean daily dose of 14.10 ± 7.17 mg for a mean duration of 38.20 ± 34.38 months. Concerning patients with growth insufficiency, the mean age was of 10.14 ± 3.35 years old: 15 boys (71.4%) versus 6 girls (28.57%). The age of the break in the growth curve in children was 10.43 ± 2.78 years, with a mean bone age of 10.52 ± 2.89 years. Other biological and radiological examinations were normal in all the patients.

The mean age for patients with sellar adenomas was of 42.64 ± 13.98 years old. The major etiology was prolactinomes in 54.5% of the cases, followed by GH adenomas in 24.2% of the cases. Nonfunctioning adenomas were found in 15.2% of the cases. The mean size of adenomas was of 14.88 ± 9.64 mm. The treatment was medical in 31.3% of the cases, surgical in 12.5% of the cases and combined in 56.2% of the cases. In all cases, the surgical procedure was trans-sphenoidal.

Evaluation of HPA axis by dynamic tests

The ITT and GST were performed on separate days after an overnight fast. Glucocorticoids were discontinued at least 24 h before testing. The maximum interval between the two dynamic tests was 7 days. Further assessment of anterior pituitary function was done by baseline hormonal testing as well as by provocative tests as required.

The ITT was performed by administering an IV bolus injection of 0.10 U/kg regular human insulin (Actrapid*). Blood samples for serum cortisol and glycaemia measurement were obtained in the basal state and at the time of symptomatic hypoglycaemia (determined by clinical findings and capillary blood glucose levels) and following 10, 20, 30, 45, 60, 90 and 120 minutes. Only patients with confirmed biochemical hypoglycaemia (a serum glucose level of ≤40 mg/dL) were included in the study. A serum cortisol level ≥200 ng/mL was accepted as sufficient responses to the ITT [9].

The GST was performed by intramuscular injection of 1 mg glucagon. Blood samples for measurement of cortisol and glycaemia were obtained in the basal state and in 30, 60, 90, 120, 150, 180 and 210 min after glucagon injection. All patients were asked to report side-effects associated with this test, and anti-emetics were administered at the discretion of the supervising physician.

Assessment of hormone levels

Serum cortisol levels were measured using the radioimmunoassay (RIA) method with a commercially available kit (Beckman Coulter) with a sensitivity of 7 ng/mL; intra-assay and inter-assay Coefficient Variation were 2.8% and 5.3%, respectively.

Group division

The study population was divided into two groups according to the value of the cortisol peak in the ITT. Group 1 (G1) included all adrenal insufficient (AI) patients with cortisol peak response <200 ng/mL. Group 2 (G2) included all patients with a normal cortisol peak response ≥200 ng/mL.

Plasma cortisol responses to both tests in groups were compared for peak value and statistical tests were performed to determine the value of cut-off cortisol in the GST.

Statistical analysis

Statistical analysis was performed using the IBM SPSS Version 23.0 (IBM Inc) program. Comparisons between the groups were carried out with two independent samples t-tests and Mann–Whitney U-tests where appropriate. Data were expressed as mean ± standard deviation. A p value <0.05 was considered as statistically
significant. Chi-square tests were used for qualitative variables. Correlation analysis was conducted with Pearson’s and Spearman’s analysis for parametric and nonparametric variables, respectively. The statistical concordance was calculated by Cohen’s Kappa index. Receiver operating characteristics (ROC) curve analysis was conducted to find a cut-off level for peak cortisol response to ITT.

**Results**

All the patients achieved hypoglycaemia (venous blood glucose level ≤40 mg/dL) during ITT. The diagnosis of the patients are presented in Table 1.

**Evaluation of the ITT**

During ITT, the mean glycemic nadir obtained at different test times was 1.70 ± 0.34 mmol/L and was found most frequently during the 30th minute (59.3%) \((p < 10^{-3})\) (Table 2). The mean baseline cortisol levels was 95.40 ± 47.08 ng/mL with a peak level of 179.75 ± 79.0 ng/mL, most commonly occurring in the 60th minute. In ITT, 44/81 (54.3%) subjects had a peak of cortisol response <200 ng/mL and were classified as Group 1 (G1). The mean cortisol peak in group 1 subjects was 122.84 ± 47.03 ng/mL significantly lower compared to a mean peak of 247.43 ± 50.61 ng/mL in Group 2 patients \((p < 10^{-3})\). Basal cortisol was significantly lower in the group 1 with 72.68 ± 33.13 ng/mL than in the group 2 with 122.43 ± 47.26 ng/mL \((p < 10^{-3})\). The cortisol responses for both groups are presented in Fig. 1.

**Evaluation of the GST**

The mean peak blood glucose level was 9.01 ± 2.03 mmol/L and was found most frequently during the 30th minute \((p < 10^{-3})\) (Table 3). The mean glycemic nadir was 4.34 ± 1.75 mmol/L and was most frequently found during the 180th minute \((p < 10^{-3})\). The mean baseline cortisol levels was 99.19 ± 54.06 ng/mL with a peak level of 182.67 ± 89.07 ng/mL, most commonly occurring in the 180th minute (38.3%). The cortisol results were grouped according to ITT classification. In the group 1, mean cortisol peak was 129.57 ± 69.53 ng/mL and it was 245.81 ± 65.67 ng/mL the group 2 \((p < 10^{-3})\) (Fig. 2).

Cortisol peak responses during ITT and GST correlated positively \((r = 0.762, p < 10^{-3})\) (Fig. 3). Individual peak values after ITT occurred relatively sooner compared to the peak values induced by GST \((50 ± 24.49\) minutes vs. \(155 ± 42.45\) minutes, \(p < 10^{-3}\)).

For the diagnosis of AI by GST, ROC analysis revealed a cut-off of 292 ng/mL with 100% sensitivity and 11% specificity (Fig. 4). In addition, ROC analysis revealed a lower cut-off of 77 ng/mL with 100% specificity and 30% sensitivity for AI. The highest proportion of correctly classified patients (86.4%) evaluated by ROC curve analysis was obtained for a glucagon-induced cortisol peak cut-off of 167 ng/mL \((\text{sensitivity}, 88.7\%; \text{specificity}, 78.5\%; \text{AUC} = 0.86; 95\% \text{confidence interval}, 0.78–0.94)\).

Considering a cut-off at 167 ng/mL, the concordance observed between the two tests would be 86.4% with a high and statistically significant k index \((k = 73.3\%; p < 10^{-3})\).

**Side effects of the GST**

The GST was well tolerated. Minor side effects occur-

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**Table 1** Type and frequency of etiologies in the population

<table>
<thead>
<tr>
<th>Etiologies</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypothalamo-hypophysary tumors</td>
<td>40.7</td>
</tr>
<tr>
<td>Growth insufficiency</td>
<td>25.9</td>
</tr>
<tr>
<td>Cranio-facial radiotherapy</td>
<td>16</td>
</tr>
<tr>
<td>Long-term corticotherapy</td>
<td>12.3</td>
</tr>
<tr>
<td>Sheehan’s syndrome</td>
<td>4.9</td>
</tr>
</tbody>
</table>

**Table 2** Glycemic means and hypoglycemia frequencies in both groups under the ITT test

<table>
<thead>
<tr>
<th>Time</th>
<th>T0</th>
<th>T10</th>
<th>T20</th>
<th>T30</th>
<th>T45</th>
<th>T60</th>
<th>T90</th>
<th>T120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycemic means (mmol/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>5.14 ± 0.94</td>
<td>3.78 ± 1.19</td>
<td>2.39 ± 0.98</td>
<td>2.55 ± 1.66</td>
<td>3.85 ± 1.97</td>
<td>4.47 ± 1.47</td>
<td>5.10 ± 1.93</td>
<td>5.26 ± 1.67</td>
</tr>
<tr>
<td>G2</td>
<td>5.01 ± 0.57</td>
<td>3.61 ± 0.75</td>
<td>2.35 ± 1.14</td>
<td>2.33 ± 1.50</td>
<td>3.76 ± 1.99</td>
<td>4.27 ± 1.41</td>
<td>4.69 ± 1.41</td>
<td>4.98 ± 1.33</td>
</tr>
<tr>
<td>Hypoglycemia frequency n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>0</td>
<td>0</td>
<td>13 (29.5)</td>
<td>26 (59.1)</td>
<td>4 (9.1)</td>
<td>1 (2.3)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G2</td>
<td>0</td>
<td>1 (2.7)</td>
<td>13 (35.1)</td>
<td>22 (59.5)</td>
<td>1 (2.7)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

G1, group 1; G2, Group 2.
Fig. 1  Mean cortisol level for each time in both groups during ITT

Fig. 2  Mean cortisol level for each time in both groups during GST
red in 35 patients (43.2%). The mean age of patients with adverse events was 42.89 ± 19.75 years with extremes ranging from 8 to 79 years old. The most common symptoms were nausea (24%) and vomiting (22.16%). Of these patients, 6 (7.4%) received antiemetic (Metoclopramide). The times of occurrence were between 90 and 120 minutes of the test. No patients had symptomatic hypoglycemia.

**Discussion**

To our knowledge, this is the first case series evaluating the use of GST in inducing cortisol secretion in Africa. The diagnosis of central adrenal insufficiency can be difficult, particularly in subjects with hypothalamic-pituitary diseases and partial ACTH deficiency, or in those with recent pituitary surgery or brain irradiation when the adrenal cortex may still be responsive to stress [10]. Although ITT has been validated against a response to surgical stress and is considered the reference standard test for evaluating the integrity of the HPA [11, 12], it is contraindicated in infants and in patients with cardiovascular disease or a history of seizures [13]. For these reasons, alternative tests including low-short ACTH, standard ACTH, metyrapone and glucagon testing have all been proposed [10, 14-16].

**Table 3** Glycemic means and frequency of hyperglycemia and hypoglycemia in both groups under the GST

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>T0</th>
<th>T30</th>
<th>T60</th>
<th>T90</th>
<th>T120</th>
<th>T150</th>
<th>T180</th>
<th>T210</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycemic means (mmol/L)</td>
<td>G1</td>
<td>5.35 ± 1.15</td>
<td>8.27 ± 1.54</td>
<td>8.01 ± 2.17</td>
<td>6.94 ± 2.86</td>
<td>5.84 ± 2.80</td>
<td>5.11 ± 2.17</td>
<td>5.03 ± 1.95</td>
</tr>
<tr>
<td></td>
<td>G2</td>
<td>5.35 ± 1.28</td>
<td>8.55 ± 1.67</td>
<td>7.94 ± 2.80</td>
<td>6.32 ± 3.15</td>
<td>5.39 ± 2.52</td>
<td>4.99 ± 1.99</td>
<td>4.85 ± 1.69</td>
</tr>
<tr>
<td>Hyperglycemic peaks frequency n (%)</td>
<td>G1</td>
<td>0</td>
<td>25 (56.8)</td>
<td>11 (25)</td>
<td>7 (15.9)</td>
<td>1 (2.3)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>G2</td>
<td>0</td>
<td>21 (56.8)</td>
<td>13 (35.1)</td>
<td>3 (8.1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hypoglycemic peaks frequency n (%)</td>
<td>G1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 (2.3)</td>
<td>6 (13.6)</td>
<td>15 (34.1)</td>
<td>14 (31.8)</td>
</tr>
<tr>
<td></td>
<td>G2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6 (16.2)</td>
<td>9 (24.3)</td>
<td>6 (16.2)</td>
<td>9 (24.3)</td>
</tr>
</tbody>
</table>

G1, Group 1; G2, Group 2.

**Fig. 3** Individual cortisol peak levels during ITT in 81 patients plotted against cortisol peaks during GST

Cortisol cut-points under glucagon
The physiological mechanisms by which glucagon stimulates the cortisol secretion are still poorly explained [15, 17]. The glycemic fluctuation, which stimulates the synthesis of cortisol during the late fall in blood sugar, are among the most frequently mentioned hypothesis [18]. The results of our work argue in favor of this physiological mechanism, because we found an initial decrease of cortisolemia at the early times of the test associated with hyperglycemia under glucagon, then a significant response of concomitant cortisol with glycemic nadirs at late times. Another mechanism by which glucagon may possibly stimulate cortisol secretion is via activation of the central noradrenergic pathways [19]. Although glucagon has been shown by Goodwin et al. to induce noradrenaline release in healthy subjects who finds two peaks of norepinephrine preceding the peaks of cortisol in the 30th minute and the 150th minute [20]. This release by itself facilitates GH secretion and helps in the secretion of cortisol [10, 18].

The cortisol peak after glucagon was observed between 150 and 180 min, whereas the cortisol peak after ITT was observed between 45 and 60 min. The blood glucose peak recorded in our patients 60 min after glucagon was followed by a decline in glucose concentrations at 90 min, with a late rise that are similar to other studies [10, 15, 21]. Hence, we propose that the shortened GST can be used without losing its diagnostic utility, which could simplify the test in clinical practice reducing costs and resources as many authors may suggest [15]. The mean cortisol peak after glucagon was similar to that observed after ITT, and there was a significant correlation between cortisol response to glucagon and that to ITT. Our results are consistent with the work of Andler et al. [22] as well as those of Littley et al. [19] who did not show a difference between the peaks of cortisolemia between the two tests. We found a positive correlation for cortisol peaks in both tests as Spathis et al. [6] objected with a correlation (r = 0.849). Other studies found significantly lower cortisol peaks during GST than during ITT [15].

In the present study, the ROC curve analysis identified the best diagnostic accuracy for a peak cortisol to glucagon of 167 ng/mL with an AUC of 0.86, demonstrating that glucagon, although apparently less accurate than ITT, is nonetheless a reliable test for the diagnosis of central adrenal insufficiency. The determination of cortisol cut-off is variable according to the teams and methods of testing and dosing [3, 8, 15, 21]. Cut-off reported in the literature are not consensual showing variability in sensitivity and specificity. Of all the determined values, the cut-off of cortisol under GST was always lower than that of cut-off under ITT but variable according to the authors. In a recent study, peak cortisol response of 108 ng/mL to GST was suggested to be the minimum acceptable cut-off value [3]. By comparing CRH testing and GST in children, Boettner et al. suggested a cut-off of 163 ng/mL with a sensitivity of 89% and specificity of 87% [21].

In our study, there was a good concordance between ITT and GST. Furthermore, although it is known that normal individuals may have subnormal cortisol responses to GST so the ability to predict an adrenal crisis has not been clearly demonstrated [23, 24]. Because of its good concordance, some authors confirm the superiority of glucagon as a first-line test [15, 21, 25]. It may be superior to synacthen test because of its disadvantages which are the poor response of the corticotropic axis in post-stroke pituitary (pituitary surgery: must be delayed from 4 to 6 months, radiotherapy: delayed from 9 to 12 months), poor sensitivity in case of damage partial corticotropic axis and exclusive stimulation of the corticotropic axis [14, 26].

Prior GST studies have reported that the rates of side effects ranged from 15 to 50% [17, 27], with nausea
being the commonest. In our study, nausea occurred in 26.62% of the cases, mainly between 90 and 150 min. Other side-effects such as vomiting has also been reported in 10 to 34% of patients [18] that are compatible with our findings. Importantly, all side-effects resolved by 210 mins of the test. Other reported side-effects included hunger, headaches and abdominal cramping that occurred mainly between 60 and 210 min as reported in other studies [8]. The stopping of the test is exceptional, and there were no stopping in our study. In some series, there was a break of the test in patients for poorly tolerated hypoglycaemia [28].

**Conclusion**

The evaluation of the corticotropic axis according to the various guidelines benefits from several dynamic tests of utility and of variable superiority. Since ITT is the gold standard, its indications have decreased in recent years because of its numerous contraindications to be replaced by the synacthen test, which allows a cortisol evaluation of a sensitivity and specificity comparable, without side effects. The usefulness of the Glucagon test has been evoked for a long time, and its accuracy in the evaluation of the corticotropic axis leaves no doubt in view of the many international studies and consensus that validate and recommend it. Due to its low side effects and the possibility of coupled dosing with GH, the glucagon test is a valid alternative for a contraindication to the insulin hypoglycaemia test. It also allows evaluation of early hypothalamic-pituitary disorders without response variability, which remains a valuable advantage over synacthen test.

**Compliance with Ethical Standards**

**Disclosure of potential conflicts of interest**

On behalf of all authors, the corresponding author states that there is no conflict of interest.

This manuscript was written without any financial support.

**Informed Consent**

A written informed consent was obtained from all the patients before the beginning of the study.

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