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High Prevalence of Hypovitaminosis D in Postmenopausal Women with Type 2 Diabetes Mellitus

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Abstract: The link between vitamin D and type 2 diabetes mellitus (T2DM) is intensively studied. This study aims to define the serum concentration of 25-hydroxyvitamin D (25-OH D) and to investigate the relationship between 25-OH D status, glycated hemoglobin (HbA1c) and body composition in postmenopausal women with T2DM and in non-diabetic controls. In this cross-sectional study, 75 women with T2DM and 32 control subjects were selected. Serum 25-OH D, intact parathyroid hormone (PTH), calcium, fasting glucose and HbA1c, were measured. The mean 25-OH D level was 21.4 ± 11.4 ng/ml (range 4.1–50.7 ng/ml) in diabetic women and 30.3 ± 9.4 ng/ml (range 10.8–54.2 ng/ml) in control group ($p<0.001$). The prevalence of hypovitaminosis D (< 30 ng/ml) was higher in vitamin D_3 non-supplemented T2DM women (89% vs. 63% controls); the difference diminished in vitamin D_3 (500–1000 IU per day) supplemented subgroups (45% diabetics vs. 42% controls). In T2DM women, 25-OH D levels were not associated to HbA1c, duration of diabetes, fasting glucose and PTH levels, however, 25-OH D levels negatively associated with body mass index ($p=0.011$), total body fat mass ($p=0.005$) and total body lean mass ($p=0.004$). The prevalence

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of hypovitaminosis D is higher in non-supplemented postmenopausal women with T2DM than in non-diabetic controls (89% vs. 63%). Obesity is a risk factor for vitamin D insufficiency in T2DM postmenopausal women. Further studies evaluating relationships between fat, muscle, bone and vitamin D metabolism in T2DM patients are warranted.

Introduction

The effect of vitamin D on bone tissue and calcium-phosphate homeostasis is well known. Vitamin D deficiency may lead to osteoporosis, osteomalacia and is associated with diffuse muscle pain and muscle weakness and increased risk of falls (Plotnikoff and Quigley, 2003; Bischoff-Ferrari et al., 2004). In addition, vitamin D deficiency has been linked to a broadening field of health problems including several types of cancer and autoimmune or metabolic diseases such as type 1 diabetes mellitus and type 2 diabetes mellitus (Wolden-Kirk et al., 2011).

Type 2 diabetes mellitus (T2DM) is a progressive chronic disease recognized by both insulin resistance and β-cell dysfunction (Badawi et al., 2014). There is evidence that patients with T2DM have an increased risk of fractures (Janghorbani et al., 2007; Martinez-Laguna et al., 2015). However, despite the increased fracture risk, bone mineral density (BMD) is generally higher in patients with T2DM (Vestergaard, 2007). Additional skeletal material aspects, such as accumulation of advanced glycation end products (AGEs) that are undetectable by BMD may contribute to diabetic skeletal fragility. As the incidence of T2DM continues to increase, it is necessary to understand what stands behind the increased fracture risk in these patients.

The reported prevalence of vitamin D deficiency or insufficiency in patients with T2DM varies from 70 to 90% (Tahrani et al., 2010; Miñambres et al., 2014; Muscogiuri et al., 2016) and depends on the threshold used to define vitamin D deficiency or insufficiency. The risk factors for vitamin D insufficiency in T2DM include poor dietary habits, lack of sun exposure, obesity, renal impairment and genetic predisposition (Penckofer et al., 2008).

The underlying mechanism explaining the association between vitamin D deficiency and T2DM is not fully understood. Several explanations have been proposed. Norman et al. (1980) identified expression of the vitamin D receptor (VDR) in rat pancreatic cells and demonstrated that a deficiency of vitamin D inhibits the production of insulin. In addition, the deficiency of vitamin D has been implicated as the predictive factor for the occurrence of diabetes (Scragg et al., 2004) and increasing the vitamin D concentration in the blood has a positive effect on maintaining glucose homeostasis by increasing insulin sensitivity (Delvin, 2011).

The aim of this study is to define the serum 25-hydroxyvitamin D (25-OH D) levels and to investigate the relationship between 25-OH D status, glycated hemoglobin and body composition indices in postmenopausal women with T2DM and non-diabetic controls.

Methods

Postmenopausal women with T2DM on anti-diabetic medication or newly detected T2DM, who attended a preventive bone mineral density (BMD) measurement, were considered for the study. The study duration was from October 2012 till October 2013. Exclusion criteria for patients were abnormal serum calcium level, serum creatinine level > 110 μmol/l, estimated Glomerular Filtration Rate (eGFR) < 1 ml/s/1.73 m^2 and proteinuria, diseases other than osteoporosis and T2DM that would interfere with bone metabolism such as primary hyperparathyroidism, liver disease, malabsorption, medical history of diabetic nephropathy; or use of any other medication affecting bone metabolism within the 3 years prior the selection, such as bisphosphonates, raloxifene, strontium ranelate, fluoride, glucocorticoids, thiazolidinedions, hormone replacement therapy or vitamin D_3 supplements (in a higher dose than 1000 IU per day). A total of 75 postmenopausal women with T2DM (mean age 66 ± 8.5 years) were eligible for the analysis. A total of 20 patients were supplemented with a low dose of vitamin D_3 (oral supplementation of vitamin D_3 with 500–1000 IU per day), 55 patients did not take any vitamin D_3 supplements. The majority of T2DM patients were treated by metformin (n=35); 18 patients were treated by combination of metformin with gliptins, sulfonylurea derivatives or insulin; 4 patients were taking sulfonylurea derivatives, 2 patients were treated by insulin and 16 patients with newly detected T2DM were without T2DM treatment.

The control group included postmenopausal women without T2DM who attended a preventive bone mineral density (BMD) measurement. The same exclusion criteria were respected also for the control group. A total of 32 postmenopausal women (mean age 64.1 ± 5.2 years), were eligible for the analysis. A total of 12 control subjects were provided with a low dose of vitamin D_3 supplements (oral supplementation of vitamin D_3 with 500–1000 IU per day), 20 patients did not take any vitamin D_3 supplements.

The study was undertaken with the understanding and written consent of each subject, with the approval of the Ethics Committee of the General University Hospital, and within compliance of the National Legislation and the Code of Ethical Principles for Medical Research Involving Human Subjects of the World Medical Association (Declaration of Helsinki).

Bone mineral density measurement

The BMD was determined using a dual energy X-ray absorptiometry (DXA) densitometer (Discovery A, Hologic, Inc., MA, USA, Software vision: Apex 3.0). BMD was measured at the lumbar spine (L1-L4), as well as the total femur, femoral neck and the whole body in all participants. We measured body-composition variables from the whole body scan.

Laboratory analysis

Routine biochemical analysis was performed with fresh samples; other aliquots were stored at –70 °C before being analysed. The serum calcium levels were measured by standard automated analytical procedure (Modular; Roche Diagnostics, Germany). The serum concentrations of intact PTH (parathyroid hormone) and 25-OH D were measured using electrochemiluminescence-based immunoanalysis (Modular; Roche Diagnostics, Germany). The serum concentrations of 25-OH D were considered as deficient (< 10 ng/ml), insufficient (10–30 ng/ml) or normal (≥ 30 ng/ml). The serum glycated hemoglobin (HbA1c) concentrations were assessed by high performance liquid chromatography. The serum fasting glucose was measured by using the enzymatic colorimetric (GOD-PAP) method.

Statistical analysis

Data were expressed by means and standard deviations if not otherwise stated. *T*-test was used for comparisons of clinical and biochemical characteristics between groups. The associations were analysed using multiple linear regression analysis. The significance was reached with a p-value < 0.05. Statistical analyses were made using SigmaStat statistical software v.3.5 (Jandel, San Rafael, USA).

Results

The demographic data and patients characteristics are stated in Table 1. In this cross-sectional study, T2DM postmenopausal women had significantly lower 25-OH D levels (the mean 21.4 ± 11.4 ng/ml) when compared to controls (the mean 30.3 ± 9.4 ng/ml) ($p<0.001$) and significantly higher PTH level in T2DM patients versus controls ($p=0.026$). Postmenopausal women with T2DM had significantly higher BMI (body mass index), total body fat mass, total body lean mass as well as total femur BMD when compared to control subjects (Table 1). The prevalence of obesity was 53% in T2DM postmenopausal women and 13% in control group.

In subjects without vitamin D_3 supplementation, the prevalence of hypovitaminosis D (< 30 ng/ml) was higher in T2DM postmenopausal women than in non-diabetic controls (89% vs. 63%). Diabetic non-supplemented postmenopausal women had significantly lower 25-OH D levels (the mean 18.3 ± 9.2 ng/ml) than the control group (the mean 27.99 ± 8.6 ng/ml) ($p<0.001$) (Table 2A).

In vitamin D_3 (500–1000 IU per day) supplemented subjects, hypovitaminosis D was seen in 45% of T2DM patients and 42% of control subjects. No statistically significant difference was found between 25-OH D levels in diabetic supplemented postmenopausal women versus supplemented control group (Table 2B).

In whole group of subjects, there was no significant difference in glycated hemoglobin, fasting glucose or duration of diabetes between T2DM postmenopausal women with hypovitaminosis D and T2DM postmenopausal women with normal 25-OH D levels. T2DM postmenopausal women with

Table 1 – Demographic data and patients characteristics

	Postmenopausal T2DM women	Controls	P-value
N	75	32	
Age (years)	66 ± 8.5	64.1 ± 5.2	ns
Years after menopause (years)	16.6 ± 8.3	16.1 ± 5.0	ns
BMI (kg/m^2)	31.8 ± 6.7	25.8 ± 4.0	<0.001
BMD LS (T-score)	–0.95 ± 0.15	–1.18 ± 0.8	ns
BMD total femur (T-score)	–0.27 ± 1.13	–0.74 ± 0.7	0.027
BMD femoral neck (T-score)	–1.13 ± 1.1	–1.3 ± 0.6	ns
Whole body BMD (T-score)	–0.56 ± 1.5	–0.52 ± 1.2	ns
Total body fat mass (kg)	34.8 ± 11.1	25.4 ± 6.5	0.002
Total body lean mass (kg)	47.9 ± 7.3	41.3 ± 5.0	0.001
S-25-OH vitamin D (ng/ml)	21.4 ± 11.4	30.3 ± 9.4	<0.001
S-intact PTH (pmol/l)	5 ± 2.2	4 ± 1.5	0.026
S-calcium (mmol/l)	2.3 ± 0.1	2.27 ± 0.1	ns
S-glycated hemoglobin A_{1c} (HbA_{1c}) (mmol/mol)	52.2 ± 15.7	38.1 ± 3.6	<0.001
S-fasting glucose (mmol/l)	7.1 ± 1.9	5 ± 0.5	<0.001
S-creatinine (μmol/l)	67.99 ± 13.6	70.64 ± 9.3	ns
GFR (ml/s/1.73 m^2)	1.21 ± 0.21	1.17 ± 0.13	ns

T2DM – type 2 diabetes mellitus; 25-OH D – 25-hydroxyvitamin D; BMI – body mass index; BMD – bone mineral density; LS – lumbar spine; S – serum; PTH – parathyroid hormone; GFR – Glomerular filtration rate

Table 2A – Definition of vitamin D status in T2DM postmenopausal women and controls without vitamin D_3 supplementation

	Non-supplemented T2DM women	Non-supplemented controls	P-value
N	55	20	
Age (years)	65.6 ± 9	64.0 ± 5.9	ns
BMI (kg/m^2)	31.7 ± 7	26.9 ± 4.2	0.003
Total fat mass (kg)	34.5 ± 11	26.6 ± 6.8	0.029
Total lean mass (kg)	48.2 ± 7	41.5 ± 5.6	ns
S-25-OH vitamin D (ng/ml)	18.3 ± 9.2	27.99 ± 8.6	<0.001
Vitamin D deficiency (< 10 ng/ml)	10 (18%)	0 (0%)	
Vitamin D insufficiency (10–30 ng/ml)	39 (71%)	12 (63%)	
Normal vitamin D values (≥ 30 ng/ml)	6 (11%)	7 (37%)	

T2DM – type 2 diabetes mellitus; 25-OH D – 25-hydroxyvitamin D; BMI – body mass index

hypovitaminosis D had significantly lower serum calcium levels when compared to T2DM postmenopausal women with normal 25-OH D levels (Table 3).

In postmenopausal women with T2DM, the 25-OH D levels were not associated with HbA1c, duration of diabetes, fasting glucose and PTH levels. Serum 25-OH D level negatively associated with total body fat mass (p=0.005, Figure 1A), total body lean mass (p=0.004, Figure 1B) and body mass index (p=0.011, Figure 2A).

Table 2B – Definition of vitamin D status in T2DM postmenopausal women and controls supplemented with vitamin D_3

	Supplemented T2DM women	Supplemented controls	P-value
N	20	12	
Age (years)	67.0 ± 7	64.08 ± 5.9	ns
BMI (kg/m^2)	31.8 ± 5.6	23.88 ± 2.7	<0.001
Total fat mass (kg)	35.5 ± 10.1	21.7 ± 3.7	0.022
Total lean mass (kg)	46.9 ± 7.5	40.4 ± 2.4	ns
S-25-OH vitamin D (ng/ml)	29.8 ± 12.8	34.2 ± 9.8	ns
Vitamin D deficiency (< 10 ng/ml)	1 (5%)	0 (0%)	
Vitamin D insufficiency (10–30 ng/ml)	8 (40%)	5 (42%)	
Normal vitamin D values (≥ 30 ng/ml)	11 (55%)	7 (58%)	

T2DM – type 2 diabetes mellitus; 25-OH D – 25-hydroxyvitamin D; BMI – body mass index

Table 3 – Glucose metabolism parameters and body composition indices in vitamin D deficient T2DM postmenopausal women versus T2DM postmenopausal women with normal vitamin D concentration

	Normal vitamin D concentration	Vitamin D deficiency/ insufficiency	P-value
N	17	58	
Age (years)	69.9 ± 8.4	64.9 ± 8.3	0.036
Years after menopause (years)	20.1 ± 8.4	15.5 ± 8.1	0.050
BMI (kg/m^2)	29.1 ± 4.1	32.6 ± 7.1	ns
Total fat mass (kg)	28.6 ± 9.9	36 ± 11.0	ns
Total lean mass (kg)	44.4 ± 8.9	48.6 ± 6.9	ns
S-calcium (mmol/l)	2.35 ± 0.1	2.28 ± 0.1	0.005
S-25-OH vitamin D (ng/ml)	38 ± 6.6	16.5 ± 7.1	<0.001
S-intact PTH (pmol/l)	4.8 ± 1.6	5.1 ± 2.3	ns
S-fasting glucose (mmol/l)	7.5 ± 2.3	7 ± 1.7	ns
T2DM duration (years)	5.1 ± 6.2	6.6 ± 6.7	ns
S-glycated hemoglobin A_{1c} (HbA_{1c}) (mmol/mol)	53.1 ± 11.9	51.9 ± 16.6	ns

T2DM – type 2 diabetes mellitus; 25-OH D – 25-hydroxyvitamin D; BMI – body mass index; PTH – parathyroid hormone

Furthermore, the serum 25-OH D level was negatively associated with total proximal femur BMD (p=0.014) in T2DM patients. Total proximal femur BMD positively associated with BMI (p<0.001), total body fat mass (p<0.001) and also total body lean mass (p<0.001) in T2DM patients. These associations persisted after adjustment for age and duration of T2DM. In a control group, the 25-OH D levels were associated with body mass index (p=0.020, Figure 2B).

The PTH levels were not associated with HbA1c, duration of diabetes, fasting glucose, BMI, as well as body composition indices in T2DM postmenopausal women.

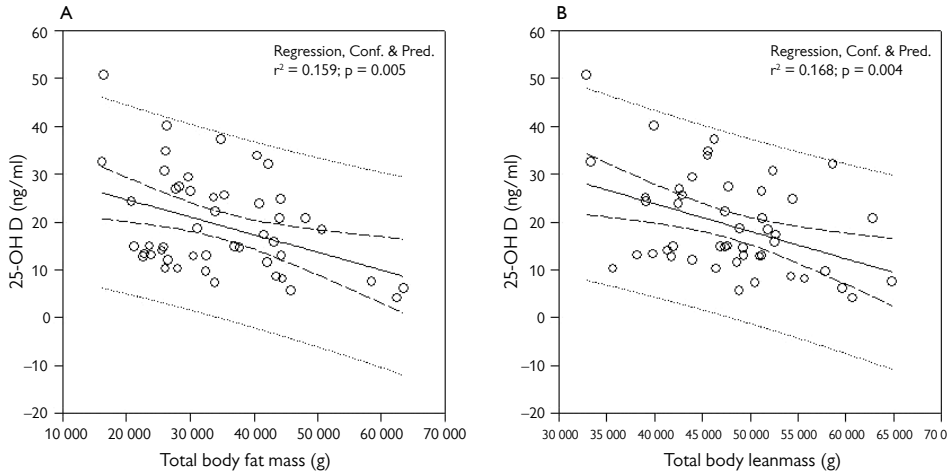

Figure 1A and B – Relationship between total body fat mass (A) or total body lean mass (B) and 25-hydroxyvitamin D (25-OH D) in type 2 diabetes mellitus (T2DM) patients. Dotted lines: prediction intervals.

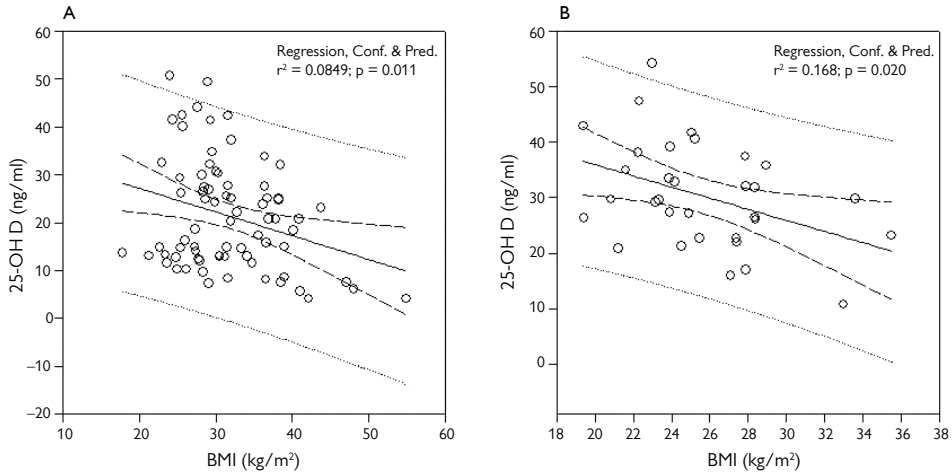

Figure 2A and B – Relationship between body mass index (BMI) and 25-hydroxyvitamin D (25-OH D) in type 2 diabetes mellitus (T2DM) patients (A) and controls (B). Dotted lines: prediction intervals.

No association between PTH and BMI or body composition indices was found also in the control group.

Discussion

Prevalence of hypovitaminosis D

Vitamin D deficiency is recognized as a worldwide issue (Personne et al., 2013). The estimated prevalence of vitamin D insufficiency in the general population is as high as 50 to 80% (Holick et al., 2005; Ginde et al., 2009). The reported prevalence

of vitamin D deficiency or insufficiency in patients with T2DM depends on the threshold used to define vitamin D deficiency or insufficiency. In our study, vitamin D insufficiency was defined as a 25-OH D level < 30 ng/ml, which is in accordance with the recommendation of International Osteoporosis Foundation (IOF).
Our data showed higher prevalence of hypovitaminosis D in non-supplemented postmenopausal women with T2DM than in non-diabetic controls (89% vs. 63%). These data are consistent with a previously published study, in which 74.6% of patients with T2DM had hypovitaminosis D (Miñambres et al., 2014). In another study, the prevalence of low serum 25-OH D (< 50 nmol/l) was more common in diabetics compared with controls (83% vs. 70%; p=0.07) (Tahrani et al., 2010). In a recent study the prevalence of hypovitaminosis D was higher in diabetic patients than in control subjects (90% vs. 83%; p<0.01) (Muscogiuri et al., 2016).

Hypovitaminosis D and body composition indices
Several studies have shown that patients with hypovitaminosis D had higher prevalence of overweight or obesity when compared to patients with normal 25-OH D status (Miñambres et al., 2014). Furthermore, obesity is associated with low serum 25-OH D levels (González-Molero et al., 2013; Vimaleswaran et al., 2013; Shantavasinkul et al., 2015). In our study, we found a significant negative association between 25-OH D levels and BMI (Figure 2A) and total body fat mass (Figure 1A). The exact underlying mechanism of this relationship is not clearly understood and several mechanisms have been hypothesized. One of the most discussed mechanisms is explained by low bioavailability of vitamin D when high content of body fat acts as a reservoir for lipid soluble vitamin D and increases its sequestration (Wortsman et al., 2000). Furthermore, the synthesis of 25-OH D may be decreased in obese subjects because of hepatic steatosis (Targher et al., 2007; Dasarathy et al., 2014). In addition, low sun exposure and limited cutaneous vitamin D synthesis in obese patients may also play a role (Florez et al., 2007).

Moreover, we have found a significant negative association between 25-OH D levels and total body lean mass. It has been reported in animal studies, that 25-OH D stores are distributed in the body fat and also in muscle tissue (Jakobsen et al., 2007). Therefore, it is tempting to speculate that muscle can create another reservoir of vitamin D also in humans.

In addition, our data showed no association between PTH levels and total body fat mass or BMI. These results are in contrast with previously reported data in non-diabetic subjects showing that fat mass is a significant independent determinant of serum PTH levels (Bolland et al., 2006). We suggest that this discrepancy may be caused by high variables of PTH levels.

25-OH D levels vs. PTH and bone measures
In our study, postmenopausal women with T2DM had significantly higher PTH levels when compared with non-diabetic control subjects. In the present study,

the intact-PTH level was within the normal range and 25-OH D levels were not associated with PTH levels.

Vitamin D deficiency is more common in diabetic patients with nephropathy (Usluogullari et al., 2015) which is linked to higher PTH levels. In our study, T2DM patients had no medical history of diabetic nephropathy.

However, it has been suggested, that PTH is suppressed at a lower serum 25-OH D level in obese women compared to the general population (Shapses et al., 2013). It is possible that there may be a different set point for the calcium PTH relationship in the obese, as demonstrated in a calcium-citrate clamp that showed an exaggerated PTH response to hypocalcemia as compared to normal subjects (Hultin et al., 2010; Cipriani et al., 2014). Moreover, while many subjects with hypovitaminosis D could have PTH within the "normal" reference range, they may have "functional hyperparathyroidism" (Souberbielle et al., 2003).

Vitamin D deficiency is known to be related with increased risk of fracture (Holvik et al., 2013) and positively associated with low BMD (Sadat-Ali et al., 2011). However, we have found a significant negative association between 25-OH D levels and total proximal femur BMD, but not between lumbar spine BMD and 25-OH D. The mechanism of this phenomenon might be explained by higher weight of diabetic patients, which causes the greater skeletal mechanical loading resulting in an increase in total proximal femur BMD. We have identified a significant positive association between total proximal femur BMD and BMI in T2DM patients. In harmony with these results, we have found significantly higher total proximal femur BMD in T2DM patients when compared to the control group. However, recent studies suggest that poor cortical bone quality is responsible for fragility fractures in postmenopausal diabetic women. Further techniques, such as peripheral quantitative computed tomography that enables the separate analysis of both trabecular and cortical bone compartment, may provide a better insight into the cortical bone in T2DM patients.

25-OH D level and glucose metabolism

Vitamin D deficiency is thought to influence the insulin resistance and the pathogenesis of T2DM by affecting either insulin sensitivity, β-cell function, or both (Chiu et al., 2004; Deleskog et al., 2012). However, we have found no significant difference in fasting glucose, glycated hemoglobin or duration of diabetes in group of T2DM patients with hypovitaminosis D versus patients with normal vitamin D status (Table 3). The design of our study was cross-sectional, not focused on monitoring the impact of vitamin D supplementation itself on glycemic control. Therefore, we did not measure the glycemic parameters before and after vitamin D supplementation. We have found no association between 25-OH D, PTH or body mass indices with fasting glycemia, HbA1c or duration of diabetes. Although epidemiological studies and meta-analysis showed an association between low serum 25-OH D and impaired glycaemia (Pittas et al., 2010; Mitri et al., 2011),

vitamin D intervention trials have had inconsistent results (Avenell et al., 2009; Von Hurst et al., 2010; Harris et al., 2011; Davidson et al., 2013). Therefore, it is uncertain whether vitamin D deficiency and poor glycemic control are causally interrelated or they represent two independent features of T2DM. The high prevalence of hypovitaminosis D in postmenopausal women with T2DM highlights the need for prospective studies in order to evaluate the impact of vitamin D supplementation on glucose metabolism.

Adequate dose of vitamin D

In our cross-sectional study, T2DM patients taking low doses of vitamin D_3 supplements (500–1000 IU per day) had higher 25-OH D level when compared to non-supplemented group of T2DM patients. Nevertheless, 45% of supplemented T2DM patients still had hypovitaminosis D. These data suggest that low dose vitamin D supplementation may be inadequate in T2DM postmenopausal women. Recent meta-analysis have demonstrated that serum 25-OH D concentrations less than or equal to 30 ng/ml were associated with higher all-cause mortality than concentrations greater than 30 ng/ml ($p<0.01$) (Garland et al., 2014). A meta-analysis of randomized controlled trials (non DM population) showed that supplemental vitamin D of 700–1000 IU/day reduced the risk of falls by 19% whereas achieved serum 25-OH D concentrations of 60 nmol/l or more resulted in a 23% fall reduction. No benefit was observed with lower supplemental doses or lower serum 25-OH D concentrations (Bischoff-Ferrari et al., 2009). To our knowledge, the studies dealing with the evaluation of the effect of vitamin D supplementation on BMD or risk of falls in diabetic population are missing. Based on data of from Bischoff-Ferrari and others on non-diabetic population, the serum concentrations of 25-OH D should be 75 nmol/l or more (Bischoff-Ferrari, 2007). Growing evidence suggest larger doses of vitamin D (equivalent to 2000 IU to 10000 IU daily) are required to optimise vitamin D status (Vieth et al., 2007). Furthermore, the question what dose of vitamin D should be used in obese patients to replete vitamin D stores and how to maintain normal 25-OH D levels after repletion remains unresolved (Cipriani et al., 2014).

Limitation of the study

In this cross-sectional study, we did not evaluate the duration of vitamin D_3 supplementation and patient's compliance with this supplementation. Moreover, due to low number of patients we could not validly assess the effect the seasonal fluctuations of 25-OH D levels and the effect of vitamin D_3 supplementation on glucose and bone metabolism parameters. Nevertheless, high prevalence of hypovitaminosis D (also in supplemented group) underscores the need for prospective studies to evaluate the impact of vitamin D supplementation on bone, muscle and glucose metabolism.

Conclusion

Our results demonstrate the high prevalence of low 25-OH D levels (below 30 ng/ml), affecting 89% of non-supplemented postmenopausal women with T2DM. Moreover, up to 45% of supplemented T2DM patients still have hypovitaminosis D. Obesity is a risk factor for vitamin D insufficiency in postmenopausal women with T2DM. Further especially prospective studies determining the adequate and safe dose of vitamin D, which significantly reduces the risk of fracture and affects the insulin resistance in T2DM patients are warranted.

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A Comparison of Salivary Steroid Levels during Diagnostic Tests for Adrenal Insufficiency

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Abstract: Numerous diagnostic tests are used to evaluate the hypothalamic-pituitary-adrenal axis (HPA axis). The gold standard is still considered the insulin tolerance test (ITT), but this test has many limitations. Current guidelines therefore recommend the Synacthen test first when an HPA axis insufficiency is suspected. However, the dose of Synacthen that is diagnostically most accurate and sensitive is still a matter of debate. We investigated 15 healthy men with mean/median age 27.4/26 (SD ±4.8) years, and mean/median BMI (body mass index) 25.38/24.82 (SD ±3.2) kg/m^2. All subjects underwent 4 dynamic tests of the HPA axis, specifically 1 μg, 10 μg, and 250 μg Synacthen (ACTH) tests and an ITT. Salivary cortisol, cortisone, pregnenolone, and DHEA (dehydroepiandrosterone) were analysed using liquid chromatography-tandem mass spectrometry. During the ITT maximum salivary cortisol levels over 12.5 nmol/l were found at

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60 minutes. Maximum cortisol levels in all of the Synacthen tests were higher than this; however, demonstrating that sufficient stimulation of the adrenal glands was achieved. Cortisone reacted similarly as cortisol, i.e. we did not find any change in the ratio of cortisol to cortisone. Pregnenolone and DHEA were higher during the ITT, and their peaks preceded the cortisol peak. There was no increase of pregnenolone or DHEA in any of the Synacthen tests. We demonstrate that the 10 μg Synacthen dose is sufficient stimulus for testing the HPA axis and is also a safe and cost-effective alternative. This dose also largely eliminates both false negative and false positive results.

Introduction

Diagnostic tests of the hypothalamic-pituitary-adrenal axis (HPA axis) are the subject of debate and still counter many difficulties. The gold standard for evaluating the HPA axis is considered to be the insulin tolerance test (ITT), but this test has several limitations and is not used in many countries. Another common test is the Synacthen (or ACTH) test, but various doses of the ACTH have been proposed (most commonly 1 μg or 250 μg) and there is no consensus which dose is diagnostically most accurate and sensitive. The other limitation of all tests is in evaluating total cortisol levels in serum, since only free cortisol is biologically active and critical for a tissue response.

Total cortisol includes both free cortisol and cortisol bound to albumin, corticosteroid-binding globulin (CBG) and other plasma proteins. In the non-stimulated state about 90–95% of cortisol is bound to CBG, 5–10 % to albumin with low affinity, and 5–8% exists in the free state (Torpy and Ho, 2007). Increasing and decreasing CBG concentrations in serum lead to changes in total serum cortisol levels. The free cortisol fraction remains unchanged, however, under the feedback control of the HPA axis. Despite this, changes in the concentrations of CBG and albumin have important consequences for interpretations results of clinical studies as well as for diagnosing HPA deficiencies. Changes in binding proteins may lead in miss-interpretation of results in conditions as renal insufficiency, critical illnesses, pregnancy and when using oral contraceptives (Ho et al., 2006; Qureshi et al., 2007).

For these reasons, when evaluating the HPA axis, measuring free cortisol would be preferable, since it avoids the influence of CBG (Mishra et al., 2007). Measuring of free cortisol levels in serum would have advantages because this reflects the biologically active cortisol fraction and acute changes in cortisol concentrations in the serum (Christ-Crain et al., 2007). Free cortisol fraction can be calculated, or measured in the laboratory. However, laboratory measurements are difficult and costly, and therefore are generally not performed for routine diagnostics (Vining et al., 1983; Klose et al., 2007). Other options are to use a calculated free cortisol index or calculate free cortisol according to the so-called Coolens' equation. The results, however, are often not satisfactory, since calculated values

do not reflect free cortisol levels during changes in CBG and during low albumin levels throughout a dynamic test (Christ-Crain et al., 2007; Klose et al., 2007). For this reason, alternatives are being explored, the most promising of which seems to be measuring salivary cortisol levels (Šimůnková et al., 2007, 2008; Deutschbein et al., 2009b; Perogamvros et al., 2010). A measurement of salivary cortisol shows the ultrafilterable fraction, which reflects circulating levels of the free cortisol. Some studies have recommended measuring only basal cortisol concentrations for diagnostic purposes, and while basal serum cortisol is specific for 23% of patients with adrenal insufficiency, salivary cortisol is specific for 27 % of such patients (Kazlauskaite et al., 2008; Deutschbein et al., 2009a). One of main problems is the wide variability in cortisol levels in both serum and saliva in the morning. For this reason, when an HPA axis deficiency is suspected, some authors recommend a Synacthen test rather than measuring basal cortisol concentrations (Artl, 2009).

Insufficiency of the HPA axis manifesting as adrenal insufficiency, is associated with higher morbidity and mortality. When this insufficiency is not diagnosed in time, it quickly leads to serious difficulties and sometimes even death of the patient. As shown in many studies, improperly indicated replacement therapies also carry significant metabolic risks. The ability to measure cortisol and other steroids in saliva as part of a diagnostic test of the HPA axis could make the diagnostic process easier and quicker for patients with changes in the concentration of protein-binding cortisol.

The aim of our study, therefore, was to evaluate salivary cortisol levels as well as those of cortisone, pregnenolone and dehydroepiandrosterone (DHEA) using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and chemiluminescent immunoassay (CLIA) Centaur XP Siemens in healthy volunteers, using both, the standard ITT test for diagnosing adrenal insufficiency and for comparing results with those from Synacthen tests using various concentrations of Synacthen (1 μg, 10 μg, or 250 μg).

Methods

Our study group consisted of 15 healthy men, with mean/median age 27.4/26 (SD ±4.8) years, and mean/median BMI (body mass index) 25.38/24.82 (SD ±3.2) kg/m^2. The men used no medications and had no history of using corticosteroids. All signed informed consent before starting the study, which was approved by the Ethical Commission of the Institute of Endocrinology. We performed four functional tests that are commonly used to diagnose adrenal insufficiency. The minimum time between tests was one week. The following tests were used: the 1 μg "low dose" Synacthen test (LDST), the 10 μg "medium dose" Synacthen test (MDST), the 250 μg "high dose" Synacthen test (HDST), and the insulin tolerance test (ITT). All tests were performed after an overnight fast, and were started in the morning between 7 and 9 a.m. Each dose of Synacthen and insulin were given

through a cannula inserted into the cubital vein, starting 15 minutes after cannula insertion.

Saliva was sampled at regular intervals during each test for measurements of steroid hormone levels. Saliva samples were collected in Sarstedt Salivette saliva examination tubes type 51.1534, centrifuged at 1000 rpm in the centrifuge, and frozen at –20 °C.

The details of individual tests were as follows

LDST: The contents of 1 ampule 250 μg/1 ml Synacthen (tetracosactide 250 μg, Novartis Pharma GmbH, Nuernberg, Germany) was added to 249 ml physiological solution. This dilution was prepared at the day of the test. At the beginning of the test, 1 ml of diluted solution was given intravenously, and saliva samples taken at 0, 20, 30, 40, and 60 minutes.

MDST: The contents of 1 ampule 250 μg/1 ml Synacthen (tetracosactide 250 μg, Novartis Pharma GmbH, Nuernberg, Germany) was added to 249 ml physiological solution. This dilution was prepared in the day of the test. At the beginning of the test, 10 ml of diluted solution were given intravenously, and saliva samples taken at 0, 30, 60, and 90 minutes.

HDST: At the beginning of the test, the contents of 1 ampule 250 μg/1 ml Synacthen (tetracosactide 250 μg, Novartis Pharma GmbH, Nuernberg, Germany) was given intravenously, and saliva samples taken at 0, 30, 60, and 90 minutes.

ITT: At the beginning of the test 0.1 IU per 1 kg Actrapid insulin was given intravenously. During the test blood glucose was regularly checked with a glucometer (Accu-Chek Perform), and blood pressure and pulse rate were measured every five minutes during the first hour and every ten minutes thereafter. There was a decrease in blood glucose below 2.2 mmol/l in all tests, and all patients had a spontaneous blood glucose response during the first hour followed by normalization. Saliva samples were taken at 0, 20, 30, 40, 60, 90, and 120 minutes.

Analyses of steroid levels

Cortisol, cortisone, pregnenolone, and dehydroepiandrosterone (DHEA) were measured using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) (Sosvorova et al., 2015). In addition, cortisol was also measured using the chemiluminescent immunoassay (CLIA) Centaur XP Siemens (CLIA) method.

Statistical analysis

Data were transformed by Box-Cox transformation before further processing due to non-Gaussian distribution and non-constant variance (heteroscedasticity) in all variables. Repeated-measures analysis of variance (ANOVA) was used for monitoring levels of steroids during tests. Comparison of method for determination of cortisol in saliva was performed by single regression analysis. CLIA method was selected as a reference method because it is common method

for clinical practice. The statistical software Statgraphics Centurion XVI from Statpoint Inc. (Warrenton, VA, USA) was used for data transformations, ANOVA testing.

Results

The results of salivary steroid levels measured during each test (maximum and minimum values for cortisol and cortisone) are given in Table 1.

Cortisol (Figure 1)

During the ITT test salivary cortisol levels reached a maximum of 12.5 nmol/l at 60 min. This level was exceeded during all Synacthen test doses, demonstrating sufficient stimulation during all Synacthen tests. During the LDST there was a maximum increase in salivary cortisol at 30 minutes to over 15.9 nmol/l, followed by a decrease. During the MDST there was a maximum increase at 60 minutes to over 21.1 nmol/l, then a plateau until 90 minutes. During the HDST salivary cortisol continuously increased during the 90 minutes of the test to over 24.5 nmol/l, and maximum levels likely occurred after the last sampling time.

Table 1 – Minimum and maximum levels of salivary cortisol and salivary cortisone from individual tests and times

	Time (min)	Salivary cortisol (nmol/l)	Salivary cortisone (nmol/l)
LDST	0	2.3–19.4	15.7–65.0
	20	8.8–24.2	34.1–73.3
	30	15.9–41.7	38.2–93.5
	40	17.3–36.2	39.4–79.7
	60	6.3–21.1	29.2–70.9
MDST	0	1.8–19.6	15.5–61.9
	30	16.6–46.7	33.9–104.7
	60	21.1–66.3	46.8–109.9
	90	22.2–56.6	46.6–116.3
HDST	0	3.0–14.8	22.1–56.4
	30	11.5–35.1	36.4–85.3
	60	24.4–49.4	39.0–126.6
	90	24.5–74.8	66.4–119.4
ITT	0	2.9–18.4	24.7–66.1
	20	1.2–17.7	16.2–72.7
	30	2.0–19.3	16.3–58.3
	40	2.7–36.2	17.0–59.1
	60	12.5–32.6	27.8–89.8
	90	11.2–26.3	38.5–77.4
	120	6.8–40.3	33.6–108.0

LDST – 1 μg "low dose" Synacthen test; MDST – 10 μg "medium dose" Synacthen test; HDST – 250 μg "high dose" Synacthen test; ITT – insulin tolerance test

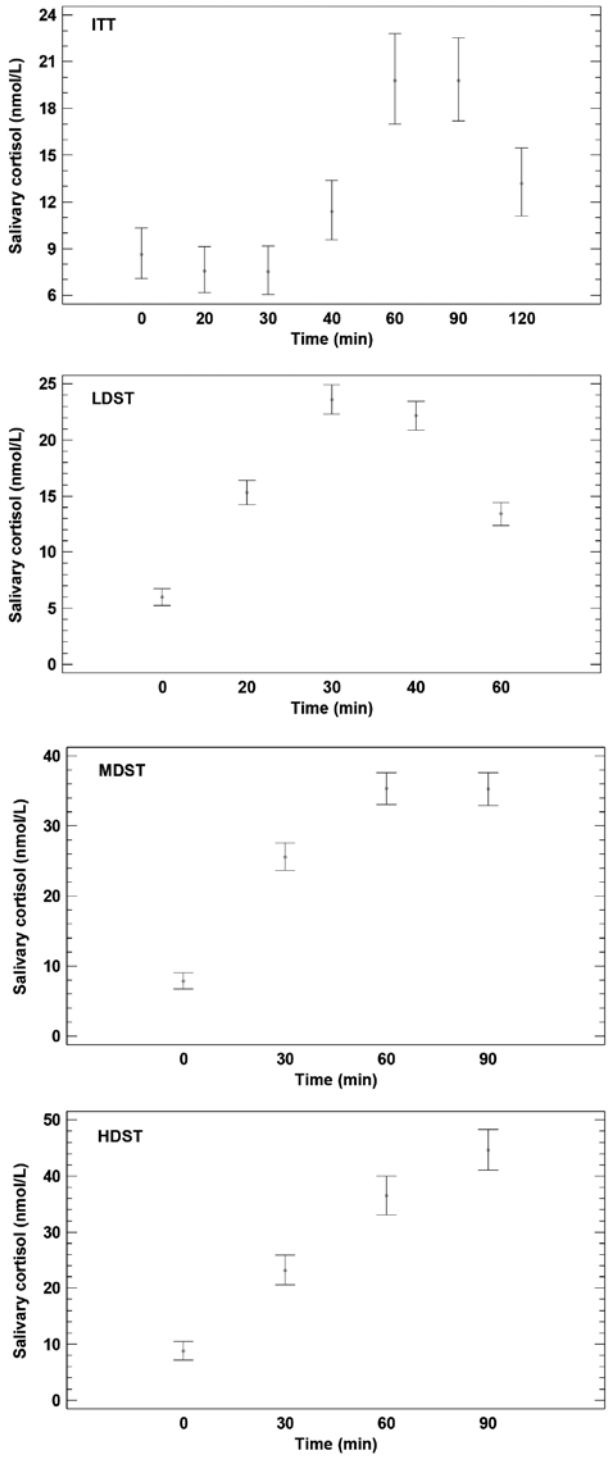

Figure 1 – *Salivary cortisol levels over time in individual tests.*

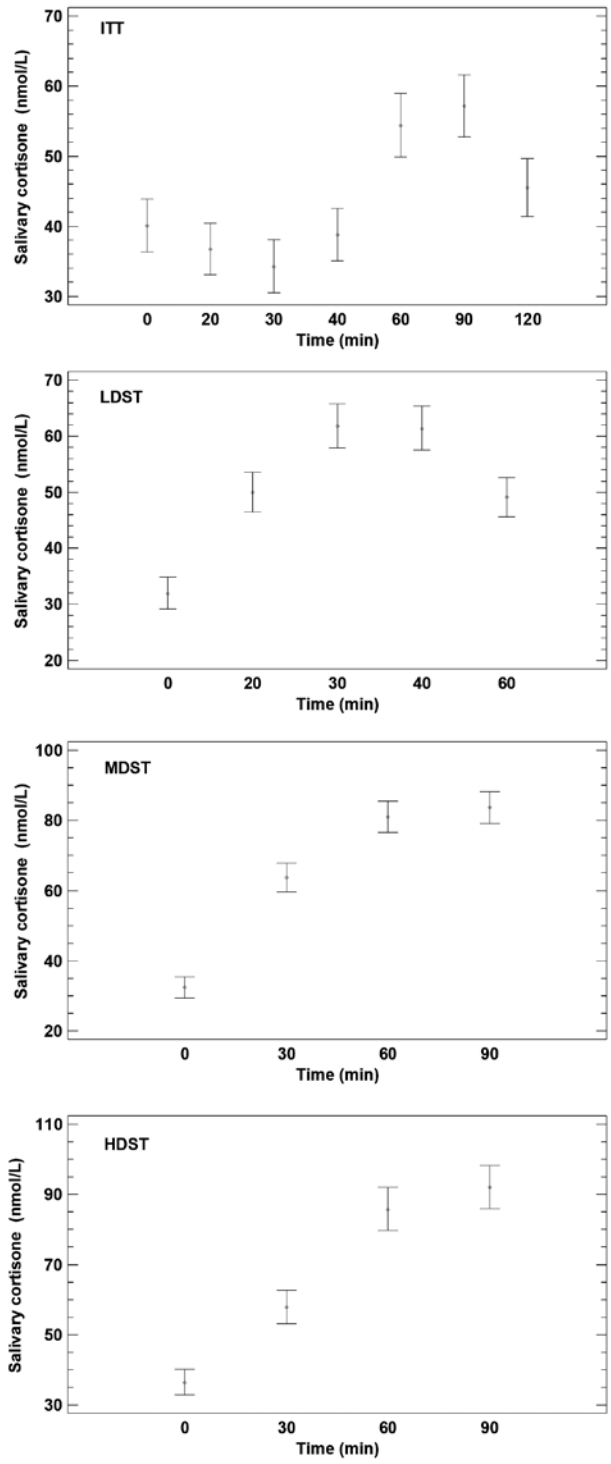

Figure 2 – Salivary cortisone levels over time in individual tests.

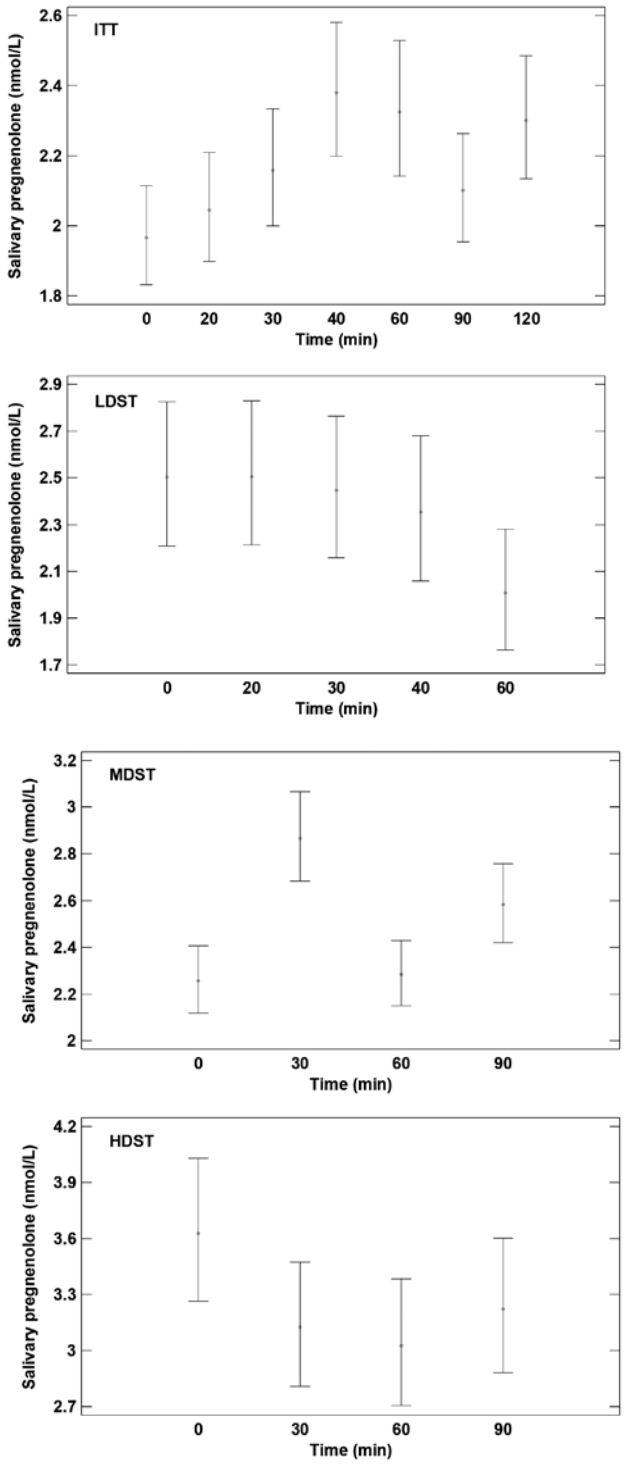

Figure 3 – Salivary pregnenolone levels over time in individual tests.

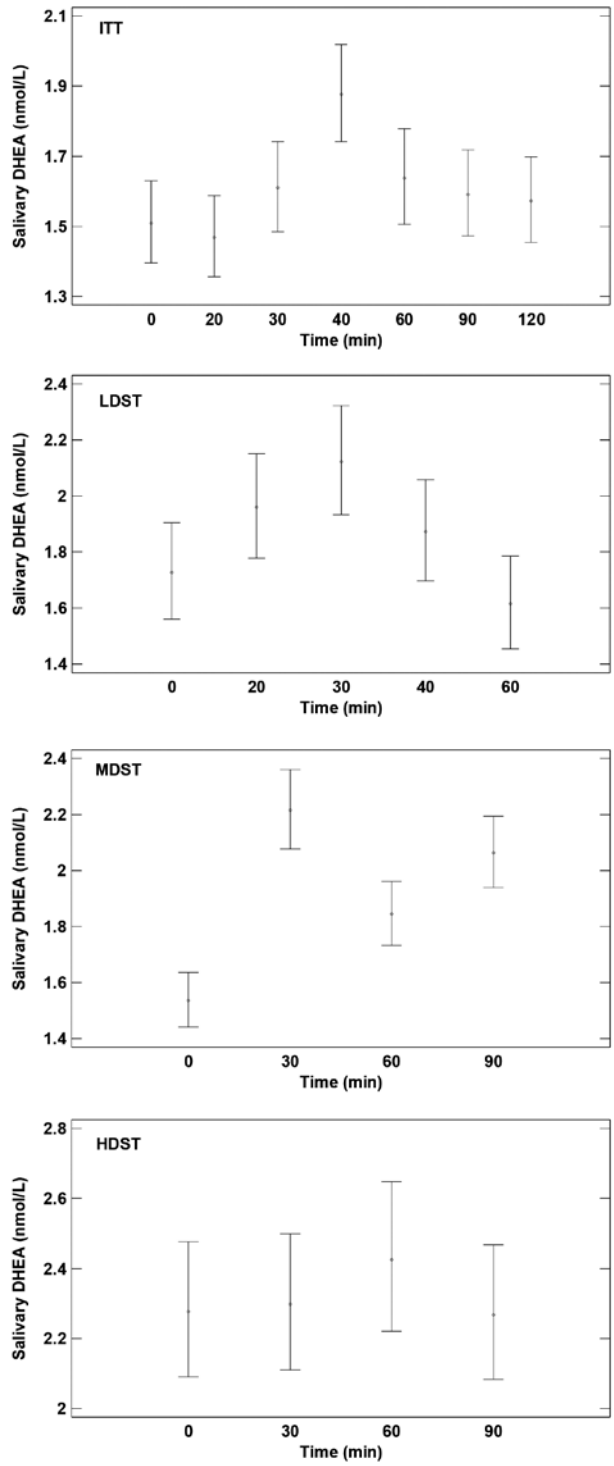

Figure 4 – Salivary DHEA (dehydroepiandrosterone) levels over time in individual tests.

Cortisone (Figure 2)
During the ITT test salivary cortisone levels reached a maximum above 27.8 nmol/l between 60 and 90 minutes. This level was exceeded during all Synacthen test doses, again demonstrating sufficient stimulation during all tests. During the LDST there was a maximum increase in salivary cortisone by 30 minutes to over 38.2 nmol/l, followed by a decline. During the MDST there was a maximum increase in 60 minutes to over 21.2 nmol/l, then a plateau until 90 minutes. During the HDST salivary cortisone continuously increased during the 90 minutes of the test to over 24.5 nmol/l, and maximum levels likely occurred after the last sampling time.

Pregnenolone (Figure 3)
During the ITT test there was an increase in salivary pregnenolone in the first 40 minutes, a reaction of the adrenal glands to hypoglycemia. There was no increase in salivary pregnenolone during any of the Synacthen tests, indicating that salivary pregnenolone levels are not appropriate for evaluating the adrenal gland response during Synacthen tests.

Dehydroepiandrosterone (Figure 4)
During the ITT test there was an increase in salivary DHEA in the first 40 minutes, a reaction of the adrenal glands to hypoglycemia. Similarly as for salivary pregnenolone, there was no increase in salivary DHEA during any of the Synacthen tests, indicating that salivary DHEA levels are not appropriate for evaluating the adrenal gland response during Synacthen tests.

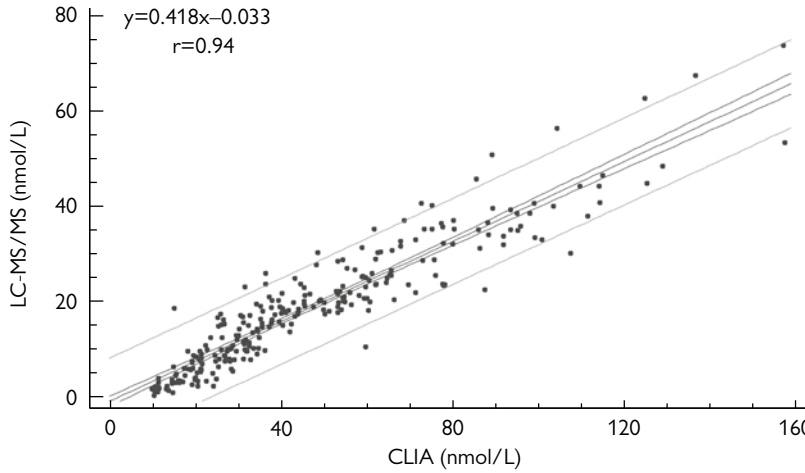

Figure 5 – Simple regression for cortisol levels. CLIA (chemiluminescent immunoassay) was used as the reference method (x) and LC-MS/MS as the test method (y). The regression line is surrounded by 95% confidence intervals (inner bounds). Outer bounds in the plot represent prediction intervals.

Comparison of the LC-MS/MS method with a chemiluminescent immunoassay (CLIA) Centaur XP Siemens

We compared cortisol levels in 300 salivary samples measured by CLIA and LC-MS/MS method (Sosvorova et al., 2015). Using CLIA as a reference there was a strong correlation between the two methods (r=0.94), with regression modelled by the equation y = 0.418x – 0.033 (Figure 5). However, the slope of the regression line indicates that CLIA considerably overestimated cortisol levels.

Discussion

All tests demonstrated a sufficient stimulus for a steroid response in saliva, with the exception of pregnenolone and DHEA in the Synacthen test. In the gold-standard ITT test for adrenal insufficiency, the salivary cortisol maximum of above 12.5 nmol/l was reached by 60 minutes. This level was exceeded during all Synacthen dose tests, demonstrating sufficient stimulation of the adrenal glands in these tests. In our healthy study population, even the 1 μg Synacthen dose induced a sufficient adrenal gland response, but the maximum cortisol concentration was reached earlier than during tests with higher Synacthen doses.

Maximum increase in serum cortisol levels during ITT tests have been reported to occur between 45 and 90 minutes (Borm et al., 2005). Maximum levels of salivary cortisol after stimulation have not been published, though recently a maximum stimulated salivary cortisol concentration of about 15 nmol/l after 250 μg of ACTH has been demonstrated (Cornes et al., 2015), which is in line with the concentrations we found during the ITT test. Our unpublished data of serum cortisol showed also the lower cortisol response in ITT likely due to overstimulation of the adrenal gland during all of the ACTH tests. In addition, other reasons of the lower maximal stimulation level of salivary cortisol during ITT compared to all of the ACTH tests may lie in alteration of salivation induced by activation of autonomic nervous system during hypoglycemia (Ekström, 1989) and relatively worse performance of saliva collection due to affected consciousness during hypoglycemia. Our subjects response to insulin did not differ significantly in their severity of obtained hypoglycaemia nor or in BMI.

Maximum serum cortisol concentrations after various Synacthen doses show a dose-dependent response (Crowley et al., 1991). We also demonstrated this dose-dependent response in salivary cortisol levels. For this reason, during lower-dose Synacthen tests, it is probably sufficient to sample saliva just during the first hour, though at more frequent intervals since the cortisol maximum may occur earlier (i.e. at 0, 20, 30, 40, and 60 minutes), as has been described for serum cortisol (Rasmuson et al., 1996; Patel and Clayton, 1999). Our results indicate that salivary cortisol during Synacthen tests behave similarly as serum cortisol levels.

The question remains, however, if stimulation in patients with altered HPA axis reactivity, for instance those with depressive symptomatology, might show false positive results (Sandström et al., 2011). This might burden patients with additional

examinations or unnecessary treatment. However, stimulation with 250 μg Synacthen is likely excessive. When testing serum cortisol levels this dose leads to repeated production of cortisol and maximum serum cortisol concentrations as long as 240 minutes after the Synacthen dosing (Crowley et al., 1991; Dicksten et al., 1997). In our study, maximum salivary cortisol levels had likely not yet been reached after 90 minutes.

As has already been described, such hyperstimulation can stimulate insufficient adrenal gland function in the subclinical phase of adrenal insufficiency and leads to false negative results, which present a high risk of underdiagnosing subclinical forms of adrenal insufficiency (Landon et al., 1984; Poršová et al., 1987; Dickstein et al., 1991). Full CBG saturation occurs at serum cortisol concentrations of 400–500 nmol/l, after which the free serum cortisol fraction changes exponentially (Torpy and Ho, 2007). During stressful situations, increased cortisol can exceed the capacity of CBG and free cortisol can increase by up to 20%, leading to marked changes in salivary cortisol concentrations (Torpy and Ho, 2007).

We suggest using a Synacthen dose of 10 μg, despite the fact that this remains a supra-physiological stimulus. A maximum cortisol response to Synacthen is achieving by administration of 12–14 pmol/l ACTH and hence the dose of 0.5 μg Synacthen is also supraphysiological (Oelkers, 1996). However, a 10 μg Synacthen dose avoids some of the limitations that arise from a 1 μg dose. It has been reported that a 1 μg Synacthen dose can lead to an insufficient response in some healthy individuals (Laureti et al., 1998, 2000, 2002). Reasons behind this might be, for instance, an incorrectly mixed or prepared dose solution, the fact that Synacthen might adhere to the plastic cannula walls, or the cannula not being properly flushed so that the entire Synacthen dose reaches the circulation. There is no unanimous method for giving Synacthen – some prefer intramuscular injections, others intravenous (Wallace et al., 2009; Chatha et al., 2010). However, it is clear that 1 μg Synacthen should only be given intravenously, since only 24% of intramuscularly injected Synacthen actually reaches the circulation (Dickstein, 1998). The possibility therefore exists when giving a 1 μg Synacthen dose that the patient does not actually receive any of the test drugs (Dickstein et al., 1991; Murphy et al., 1998; Agha et al., 2006; Wallace et al., 2009; Chatha et al., 2010).

In our results, the cortisone response in saliva mirrored that of cortisol, but reached higher concentrations in all of the tests, likely due to the activity of 11β-hydroxysteroid dehydrogenase type 2 (HSD2) in the saliva that converts cortisol to cortisone. Perogamvros et al. (2010) found that during stimulation by 250 μg Synacthen cortisone was stable, and recommended this as an alternative parameter to free cortisol when evaluating adrenal capacity. A recently published study has described a linear and bimodal correlation between cortisol and cortisone in saliva in basal levels and as well as after stimulation which agrees with the results we found in all our tests (Cornes et al., 2015). Altered 11β-HSD2 activity by genetic defects or by medication and diet (derivatives of glycyrrhetinic

acid) may lead to changes in salivary cortisol and cortisone that fact has to be taken into account and excluded (van Uum et al., 2002; Perogamvros et al., 2010).

The reaction of pregnenolone and DHEA during the tests is noteworthy. During the hypoglycemia test there were increases in both at 40 minutes, before the increase in cortisol. The ITT confirms adrenal function and the response can be seen in all steroids. This is in line with the assumption that ACTH is not a regulator of the adrenal biosynthesis of adrenal androgens, that is, mainly of that of DHEA. This applies also to pregnenolone as well. The tests with both high and low doses of Synacthen showed that there is a depletion of adrenal pregnenolone as a precursor of other hormones. The determining factor for the production of adrenal androgens has yet to be found, however.

The analyte concentrations presented in this paper were measured by LC-MS/MS. However, in routine laboratory settings immunochemical methods are most common. We compared salivary cortisol concentrations measured by both LC-MS/MS and CLIA, and found them to be highly correlated (r=0.94), however CLIA overestimated absolute values. This is in agreement with our previous results, where we compared serum cortisol concentrations using LC-MS/MS and a radioimmunoassay (RIA) (Dušková et al., 2015). The study of Vieira et al. (2014) compared salivary cortisol measured by LC-MS/MS and an in-house RIA method and also found a high correlation (r=0.82).

Results obtained when using an immunoassay also depend on the type of analyzer used. The study of Cornes et al. (2015) compared serum cortisol levels after the administration of 250 μg of Synacthen using immunoassays measured on Roche and Abbot analyzers with measurements from LC-MS/MS. Results were highly correlated, but values from the Abbott analyzer were about 20% lower than those from the Roche analyzer. The overestimation of values by immunoassays and the differences between individual analyzers all need to be taken into account when evaluating results in clinical practice. Despite this, the availability and cost-effectiveness of immunoassay methods remain the method of choice for routine clinical settings (Taylor et al., 2015).

Conclusion

Our results indicate that a 10 μg Synacthen dose is a safe and cost-effective choice when testing patients suspected of adrenal insufficiency, which also largely eliminates the risk of false positive and false negative results. In addition, the 10 μg dose can be more easily diluted in hospital pharmacies, allowing a precise dose to be given. In light of the expiration time of one month after dilution, this dose also allows the testing of as many patients as possible from one 250 μg ampule of Synacthen, making this dosing cost-effective. This issue is even more critical considering the fact that Synacthen production was recently halted for a number of months.

When evaluating test results, the methods of analysis and associated reference ranges must always be taken into account. Cortisol levels measured by different

methods may be highly correlated, but absolute values can vary by tens of percent. It is critical that each practice determine its own guidelines for a sufficient response to dynamic test of adrenal insufficiency.

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Combination of Steroids and Azathioprine in the Treatment of Ormond's Disease – A Single Centre Retrospective Analysis

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Abstract: We present a retrospective analysis of patients treated in our Department of Clinical Biochemistry, Haematology and Immunology, Na Homolce Hospital, during 1997–2013 for Ormond's disease. We analyse the clinical history, diagnostic approaches, surgical, and immunosuppressive therapies and their subsequent effect on our patients. 28 patients treated for Ormond's disease were included. Patients with established disease activity (26 patients) were given immunosuppressive treatment, using corticosteroids in combination with azathioprine. Treatment response was evaluated using clinical symptomatology, inflammatory parameters and imaging methods. In the cohort as a whole, immunosuppressive therapy was applied in 26 patients; in two patients it was not used as no inflammatory activity was found with the disease. In all 26 patients, computed tomography showed that immunosuppressive treatment resulted in partial or complete regression of inflammatory infiltrate. Out of the total number of 26 patients, two patients experienced disease exacerbation 7 and 16 months after the immunosuppressive treatment was discontinued. The longest follow-up period was 16 years; the shortest one was 21 months. Idiopathic retroperitoneal

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fibrosis – Ormond's disease – is a disease with serious complications. Standard treatment involves a combination of surgery and immunosuppressive treatment. The combination of corticosteroids and azathioprine represents a potentially safe and useful method of treatment.

Introduction

Ormond's disease – idiopathic retroperitoneal fibrosis – is a relatively rare disease with an unclear aetiology, characterised by chronic periaortitis and retroperitoneal fibrosis (RF). The inflammatory process affects the infrarenal part of the abdominal aorta and the iliac arteries, and the presence of infiltrates encasing the ureters and inferior vena cava is also commonly found. Its incidence is 1.3 in 100,000 people (van Bommel et al., 2009). This disease is currently classed as an immunoglobulin G4-related disease. The course of the disease is associated with the incidence of complications, the most serious of which are renal failure and aneurysm of the abdominal aorta or the iliac arteries. Thanks to advances in medicine, diagnostic and, in particular, therapeutic strategies are gradually changing. Improved results from pharmacotherapeutic regimens have led to a greater emphasis on early comprehensive medication therapy with corticosteroids and new immunosuppressive or immunomodulatory drugs. In our retrospective survey, we describe the clinical history, diagnostic approaches, surgical, and immunosuppressive therapies and their subsequent effect on our patients.

Patients and Methods

Patients and setting

Between 1997 and 2013 our Department of Clinical Biochemistry, Haematology and Immunology, Na Homolce Hospital, in collaboration with Department of Urology and Department of Vascular Surgery, diagnosed and subsequently treated 28 patients with idiopathic RF. The demographic data for these patients are shown in Table 1. The disease was diagnosed on the basis of a comprehensive clinical examination, using biochemical, immunological, and microbiological tests, and imaging methods including ultrasound, computed tomography (CT), positron emission tomography/computed tomography (PET/CT), and CT/arteriography. Clinical symptoms at onset were back pain, flank pain, fatigue, weight loss, fever,

Table 1 – Patient demographics and laboratory characteristics

	N	Age	CRP mg/l median	ANA positive	anti dsDNA	Rheumatoid factor	Autoimmune disease
Male	16	56.8	38.2	6 (31%)	negative	negative	1
Female	12	51.4	41.7	4 (28%)	negative	negative	7

CRP – C-reactive protein; ANA – antinuclear antibodies; dsDNA – double stranded deoxyribonucleic acid

and night sweats. Laboratory screening included a biochemistry panel, screening for hepatitis B and C, serum levels of immunoglobulin G (IgG) and the subclasses IgG1–IgG4, and the erythrocyte sedimentation rate (ESR). Patients were also examined for the presence of antinuclear antibodies, extractable nuclear antibodies, and antibodies to double-stranded DNA. RF was considered if soft-tissue density surrounded the infrarenal aorta or iliac vessels on contrast-enhanced CT and/or on histologic confirmation. Disease activity was confirmed by a PET/CT scan in 21 patients upon the initiation and discontinuation of immunosuppressive therapy. In eight patients, a biopsy sampling collection was performed percutaneously using a biopsy needle under CT navigation, whereas in one patient, laparoscopy was used to collect the sample. The diagnosis of Ormond's disease was confirmed in all the biopsied samples by subsequent histological examination. The examinations into the presence of IgG4 producing plasma cells, performed in a small number of patients (three) are not reported.

Treatment

Patients with established disease activity (26 patients) were given immunosuppressive treatment, using corticosteroids in combination with azathioprine. The initial dose of prednisone was 0.75–1 mg/kg and that of azathioprine was 2×50 mg/day. After systemic symptoms were resolved and the inflammatory markers reached negative values, corticosteroids were reduced as follows: prednisone by 10 mg a month to reach a daily dose of 10 mg; azathioprine was administered in the dose of 2×50 mg for six months and then discontinued. A follow-up CT or PET/CT scan was performed after six months of treatment. In the absence of inflammatory activity, i.e. negative biochemical parameters and a negative PET/CT scan, prednisone treatment was reduced to 5 mg/day and discontinued after two months. Where indicated, surgical procedures were applied, including ureteral stenting, nephrostomy, stenting of arteries, or arterial aneurysm surgery.

Outcomes and follow-up

The primary outcome was the reduction of clinical symptoms, extubation of obstructed ureters, and reduction of soft tissue mass, followed by the absence of inflammatory activity on a PET/CT scan. The secondary end-points included monitoring of the biochemical parameters of renal function, IgG4 concentration, and reaching normal values of inflammatory biomarkers. The patients were monitored at regular intervals of four to six weeks in the first three months when biochemical and immunological parameters were monitored. After inflammatory markers became insignificant, this interval was extended to two months. Follow-up CT or PET/CT scans were performed after the first six months and after a further three to six months in patients requiring longer treatment. The longest follow-up period was 16 years; the shortest one was 21 months.

Results

Table 1 shows the patient laboratory and demographic characteristics. Eighteen patients presented with clinical symptoms of ureteric obstruction with hydronephrosis, renal insufficiency (two patients) or renal failure (one patient). Ten cases involved patients with periaortitis, with aneurysms of the aorta and/or iliac arteries present in five of these. After laboratory testing, ten patients were found to be positive for antinuclear antibodies, without evidence of specificity for extractable nuclear antibodies. None of the patients tested positive for antibodies to double-stranded DNA. Other autoimmune diseases were present in eight patients, in which seven cases were identified as autoimmune thyroiditis and one case as Sjögren's syndrome. IgG4 concentrations were examined in 18 patients. In eight patients, an increased IgG4 concentration was found. The IgG4 concentration was within the normal range in all patients after immunosuppressive therapy. Hydronephrosis was found in 18 patients; 10 patients showed impairment of one ureter and 13 patients had impairment of both ureters. In two patients, despite an initial diagnosis of renal insufficiency, subsequent surgical intervention and immunosuppressive therapy resulted in the recovery of renal function. The standard surgical solution involved the insertion of stents, and a nephrostomy was performed on one patient. In all 26 patients, CT showed that immunosuppressive treatment resulted in partial or complete regression of the inflammatory infiltrate. After termination of the immunosuppressive therapy, 32 (89%) of the 36 ureters were successfully extubated. The presence of an abdominal aortic aneurysm was found in five patients and iliac artery aneurysms in two patients. Surgical treatment was indicated for aneurysms in four cases prior to the deployment of immunosuppressive therapy. In the case of one patient with an aneurysm, surgical treatment was not indicated after the initial diagnosis, but four months after the termination of immunosuppressive treatment, the size of the aneurysm was found to have increased and required surgery. Four patients who underwent surgery for aneurysms were subsequently given standard immunosuppressive treatment, with no exacerbation of the disease for six months after its termination. In the cohort as a whole, immunosuppressive therapy was applied in 26 patients; in two patients, it was not used as no inflammatory activity was found with the disease. In all 26 patients, a combination of corticosteroids and azathioprine was used. Out of the 26 patients, two patients experienced disease exacerbation 7 and 16 months, respectively, after immunosuppressive treatment was discontinued. The longest follow-up period was 16 years; the shortest one was 21 months. Out of the total number of 28 patients, 25 are still alive. One patient died of an acute abdominal event, and two patients died of cancer 35 and 17 months, respectively, after the discontinuation of immunosuppressive therapy.

Discussion

Idiopathic RF is a systemic disease of as yet unexplained pathogenesis. The term covers a number of common diseases – chronic periaortitis, inflammatory abdominal aortic aneurysm and perianeurysmal RF. Approximately two-thirds of RF cases are idiopathic, i.e. no primary specific cause has been identified. In this case, the condition is known as Ormond's disease. In one-third of patients, symptoms of fibrosis are found after primary malignancy, infection, or iatrogenic effects of medication (Vaglio et al., 2006). In 70% of patients, the first symptoms of the disease are recorded between 30 and 60 years of age; in idiopathic RF, men predominate in a ratio of 2:1. In typical cases where RF manifests, confirmation of the diagnosis is based on the medical history, clinical findings, and imaging results (Restrepo et al., 2011; Schmidt, 2013). Ormond's disease is a condition currently classified as an IgG4-related disease (Zen et al., 2012; Brito-Zerón et al., 2014). These disorders are characterised by increased concentrations of IgG4 in the serum and the presence of IgG4-producing plasma cells in the infiltrate. The pathognomonic significance of the antibodies produced has not yet been sufficiently determined. Published studies suggest that only a portion of patients with Ormond's disease meet this criterion (Zen et al., 2009; Takahashi et al., 2010; Laco et al., 2013). In our cohort, we found increased concentrations of IgG4 in the serum in eight patients, whereas measurements were taken from 18 patients before starting immunotherapy. Findings of elevated levels of IgG4 in the serum had no effect on the success of immunosuppressive therapy. Clinical disease presentation did not vary among patients with increased or normal IgG4 concentration. In the context of these results and in line with results from other studies (Koo et al., 2015), it would appear that not all patients with RF fall into the category of IgG4-related disease. After successful immunosuppressive therapy and remission, we found that the concentrations of IgG4 in the serum of all patients who had previously shown elevated levels had normalised. One of the complex and very important issues to be addressed in the diagnosis of RF involves distinguishing between an infectious and non-infectious aetiology of the inflammation. The emergence of a retroperitoneal or para-aortal infiltrate may also be induced by an infectious aetiology. Given the use of immunosuppressive therapy, failure to recognise this difference may have fatal consequences for the patient. Potential causative agents may include viruses (hepatitis), mycobacteria, and bacteria, i.e. *Staphylococcus aureus*, *Salmonella*, etc. (Cartery et al., 2011). In our group, we performed biopsy investigations under CT in eight cases; in one patient, we removed a biopsy specimen laparoscopically as it was not possible to remove it using a thin needle under CT. This was a patient with a suspected infectious aetiology (tuberculosis, TB) with a positive test for interferon gamma production and indications of this aetiology from the PET/CT image. Although an examination of the biopsy sample using molecular biological methods failed to confirm TB, this patient underwent anti-TB treatment along with immunosuppressive therapy. Given

the typical clinical findings, including the results of imaging methods, no biopsies were carried out on the other patients.

It is noteworthy that not all patients demonstrate disease activity using standard inflammatory markers (ESR, C-reactive protein). Here, our results are in agreement with the study by Pelkmans et al. (2012), where the negativity or positivity of acute phase reactants did not affect the final clinical outcome of patients after treatment. In our group, there were two patients for whom ESR and C-reactive protein were negative in the initial tests. Subsequent PET/CT examinations failed to find inflammatory activity in these patients. In the two patients with negative PET/CT results, we did not initiate immunosuppressive treatment, and they were routinely monitored. They currently show no increase in inflammatory activity after 2 and 3 years of follow-up, respectively. We believe that the disease can also be diagnosed in its chronic phase, when inflammatory activity may disappear spontaneously. The most valuable indication of disease activity is the PET/CT examination, which we use for each patient before deploying immunosuppressive treatment, as well as when deciding to terminate it. In this respect, it would be advantageous to find new biomarkers which could indicate the activity of the disease with a lower cost and burden placed on the patient compared with PET/CT. An example of this type of marker could be pentraxin 3 (Dagna et al., 2011).

For our patients, we used an initial dose of 0.75–1 mg of prednisone per kilogram of body weight, gradually reducing this over a treatment period of six months. After this period, we carried out PET/CT monitoring and, depending on the results, decided whether or not to extend the treatment. The maximum period of treatment was 24 months, with 5 mg/day doses of prednisone maintained over that time. Before administering azathioprin, we always carried out a pharmocogenomic assessment using thiopurine S-methyltransferase, which identified any high-risk patients (Ford and Berg, 2010).

We diagnosed our first patient with RF in 1997, and this patient has also undergone the longest follow-up period – 16 years. Of 28 patients, three died; in one case the cause was an acute abdominal disorder, with no apparent link to Ormond's disease, and two cases were due to cancer which occurred two and three years, respectively, after the termination of immunosuppressive treatment. In addition to the treatment methods referred to above, cyclophosphamide, cyclosporine A and tamoxifen are also used to treat patients with RF (Marzano et al., 2001; Binder et al., 2012; Brandt et al., 2014). Mycophenolate mofetil appears promising given its antifibrotic effects (Scheel et al., 2011). An alternative treatment for patients who do not respond adequately to treatment with corticosteroids, or for whom this therapy is associated with a higher risk, involves biological disease modifiers. These include tocilizumab, an antibody against the interleukin-6 receptor, or rituximab, a monoclonal antibody against CD20 (Maritati et al., 2012; Vaglio et al., 2013).

There are several limitations to our study: the small number of patients, the retrospective nature of the evaluation, and the absence of histopathological assessments detecting IgG4 plasma cells in the infiltrate. On the other hand, we could not find any study in the literature with this many patients treated with a combination of corticosteroids and azathioprine.

Conclusion

Idiopathic RF – Ormond's disease – is a disease with serious complications including renal insufficiency or failure and aneurysm of the abdominal aorta or iliac arteries. Its diagnosis is based on an exact diagnosis from a biopsy, or a combination of imaging methods and immunological, biochemical, and microbiological tests. Standard treatment involves a combination of surgery and immunosuppressive treatment and, provided it is applied in a timely manner, patients can expect a favourable clinical outcome.

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L1 Retrotransposons Are Transcriptionally Active in Hippocampus of Rat Brain

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Abstract: LINE1 (L1) is an autonomous, non-LTR retrotransposon and the L1 family of retrotransposons constitute around 17%, 20% and 23% in the human, mouse and rat genomes respectively. Under normal physiological conditions, the retroelements remain by and large transcriptionally silent but are activated in response to biotic and abiotic stress conditions and during perturbation in cellular metabolism. They have also been shown to be transiently activated under certain developmental programs. Using RT-PCR, we show that the L1 elements are transcriptionally active in the hippocampus region of the brain of four-month-old rat under normal conditions without any apparent stress. Twenty non-redundant LINE1-specific reverse transcriptase (RTase) sequences form ORF2 region were isolated, cloned and sequenced. Full length L1 element sequences complementary to the isolated sequences were retrieved from the L1 database. *In silico* analysis was used to determine the presence of these retroelements proximal (up to 10 kb) to the genes transcriptionally active in the hippocampus. Many important genes were found to be in close proximity of the transcriptionally active L1 elements. Transcriptional activation of the elements possibly affects the expression of the neighbouring genes.

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Introduction

Transposable elements make up sizeable components of all eukaryotic genomes, varying from 14% to over 80%. On the basis of their mechanism of transposition, they are categorized into two classes: (i) class I elements, called retrotransposons which move via an RNA intermediate by "copy-and-paste" mechanism; and (ii) class II elements, called transposons move via a DNA intermediate by "cut-and-paste" mechanism. The "copy-and-paste" mechanism implies that transposition of the element leads to increase in copy number whereas transposition by the "cut-and-paste" mechanism normally does not lead to increase in the copy number. Retroelements constitute a predominant class of elements in eukaryotic genomes and subdivided into two categories: LTR elements and non LTR elements. The mammalian genomes contain a preponderance of non-LTR retroelements. One of the transpositionally active groups of non-LTR elements in mammalian genomes is the L1 elements, LINE1 (Long Interspersed Nuclear Element). The L1 elements constitute approximately 17%, 20% and 23% in the human, mouse and rat genomes respectively (Lander et al., 2001; Waterston et al., 2002; Gibbs et al., 2004). They remain quiescent under normal physiological and developmental conditions (Furano, 2000; Ostertag and Kazazian, 2001; Moran and Gilbert, 2002); however, they could become active under environmental stress conditions (Muotri et al., 2009; Hunter et al., 2012, 2013; Terasaki et al., 2013) and certain pathological conditions (Beck et al., 2011; Kaer and Speek, 2013). The elements have shown to be active during the processes of differentiation (Muotri et al., 2005; Coufal et al., 2009), neurogenesis (Singer et al., 2010; Thomas et al., 2012), and embryogenesis (Garcia-Perez et al., 2007; van den Hurk et al., 2007; Castro-Diaz et al., 2014). Increased rate of L1 retrotransposition has also been reported in the neuronal tissues in schizophrenia and bipolar disorders (Coufal et al., 2011; Poduri et al., 2013; Bundo et al., 2014). Transpositional events in somatic cells have been shown to lead to mosaicism during neuronal differentiation (Muotri et al., 2005). Integration of the L1 elements into different locations within the genome may possibly affect the expression pattern of neighbouring genes. Activation of the elements in pathological conditions could be explained by certain degree of metabolic perturbation in the affected cells. As per the L1 database, over 11,600 full length L1 elements have been detected in human, over 6,500 in rat and over 2,000 in mouse genomes (http://line1.bioapps.biozentrum.uniwuerzburg.de/l1base.php).

L1 encodes two proteins: ORF1, an RNA binding protein and ORF2, an endonuclease and reverse transcriptase. Both the proteins are required for L1 transposition. L1 elements propagate in the host genome via an mRNA. Moran and Gilbert (2002) showed that the integrated full length L1 element is generally flanked by variably sized target site duplications. The L1 elements are thought to be transcribed by RNA polymerase II. Transcription into full-length RNA is the first step in the L1 retrotransposition that provides a template for the synthesis of

both L1-encoded proteins and a DNA copy (Furano, 2000; Ostertag and Kazazian, 2001; Moran and Gilbert, 2002). Then the reverse transcribed DNA copy may be integrated into the host genome. The increased activation of L1 retrotransposition in the neuronal genome has also been observed in mental disorders including schizophrenia and bipolar disorders (Coufal et al., 2011; Poduri et al., 2013; Bundo et al., 2014). It is conjectured that increased retrotranspositional activity of the L1 elements in schizophrenia may be triggered by genetic and/or environmental factors in the early neural development and that could be the contributing factor for the susceptibility and pathophysiology of the disease (Bundo et al., 2014).

We analysed L1 expression in different regions of brain of four-month-old rats by RT-PCR using primers designed for the L1-specific reverse transcriptase domain. We observed the presence of L1-specific reverse transcriptase (RTase) transcripts in the hippocampus under normal physiological conditions. Analysis of diversity of RTase domain of different L1 elements indicates the presence of transcript from at least 19 different L1 elements. *In silico* analysis reveals that the L1 elements active in the hippocampus are located within introns and in the close vicinity of upstream or downstream of the genes involved in neural related functions.

Material and Methods

Animals

Four-month-old male Wistar albino rats (6 for each age group) were used for all the experiments. Animals were maintained in the animal house facility of Jawaharlal Nehru University (JNU), New Delhi, at a constant temperature of 25 °C, humidity of 55% and 12 h dark and 12 h light cycle (light from 06:00 to 18:00 h). The animals were fed standard chow rat feed (Hindustan Lever Ltd., India) and water *ad libitum*. All animal experiments were approved by the JNU-Institutional Animal Ethics Committee; and all the institutional guidelines were adhered to in the care and treatment of the animals. Rats were sacrificed by cervical dislocation. Different regions of brain namely cerebral cortex, hippocampus, cerebellum, medulla and pons were excised, rapidly frozen in liquid nitrogen and stored at –80 °C.

RNA isolation and RT-PCR

Total RNA from the 4-month-old normal rat brain regions was extracted using TriZol (Invitrogen, USA) according to the manufacturer's instructions. To eliminate the possibility of genomic DNA contamination, the RNA samples were treated with the RNase free DNase I (Fermentas) according to the manufacturer's instructions. The concentration of RNA was determined and integrity of RNA in the samples was confirmed by 1.2% (w/v) MOPS agarose gel electrophoresis.

Isolation of reverse transcriptase sequences

Forward and reverse primers for PCR amplification were designed from the conserved domain within the ORF2 of the L1 element (Dobigny et al., 2004).

RT-PCR was carried out using Promega's Access RT-PCR system with slight modifications: 1 μg RNA template, 1.5 mM $MgCl_2$, and 200 nM of each of the primers (5'-ATT CTR TTC CAT TGG TCT A-3' and 5'-CCA TGC TCA TSG ATT GG-3'). The amplification was performed in a MJ Research thermocycler with the following parameters: 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 30 s, followed by a final extension step at 72 °C for 10 min. The amplified products were eluted from 1.5% agarose gel and cloned into a pGEMTeasy vector (Promega). Cloned amplicons were sequenced by Scigenome, India.

Similarity among the amplified sequences

Thirty seven different nucleotide sequences obtained were checked for non-redundant sequences and the sequences over 90% similarities were excluded. We used the Mview tool for this analysis (http://www.ebi.ac.uk/tools/msa). Mview transforms the Sequence Similarity Search results into Multiple Sequence Alignment (Brown et al., 1998). Of all the sequences, 20 non-redundant sequences were used for further analysis.

Distribution of L1 elements on different chromosomes

The sequence of each chromosome was analysed using Ensembl BLAST/BLAT (Hubbard et al., 2002) online program (http://asia.ensembl.org/Multi/blastview). BLAT analysis was performed with "exact matches" search sensitivity. Top 1,000 results for each sequence were sorted by raw score. Out of all results only those alignments were selected which showed approximately 95% sequence similarity and 90% length match with query sequences. Total number of alignments per chromosome were calculated and converted to percentages for each query sequence. To show the relationship between L1 density and chromosome size, Pearson's correlation test was performed. The statistical analyses and graphs were built using Prism GraphPad v5.

Location of genes in the vicinity of intact L1 elements

Each chromosomal sequence was submitted to the L1 database (http://line1.bioapps.biozentrum.uni-wuerzburg.de/l1base.php) to know intact L1 elements-associated sequences within the rat genome (using RGSC v3.4-Ens30.34; 6108 Entries, Last Update: 2007-10-14 21:07:58). The first top 100 were sorted by intactness score, which also provided information about genes associated with each resultant L1 element. Each L1 UID was selected and checked for the vicinity of genes belonging to a specified probable chromosomal location using Ensembl Rat Genome Database RGSC v3.4. The chromosomal location was ascertained in "Ensembl Genome Browser" (http://asia.ensembl.org/index.html) in its required input format (e.g. Chromosomal Position-7:105925414-105916554). Only those genes were selected for further analysis, which were in the direct vicinity i.e. located within the gene

or located up to 10 kb from the upstream or downstream to an annotated protein coding gene. Thus, data were rearranged manually to show the presence of intact L1 element in the vicinity of a gene (upstream or downstream or intragenic). The results of Ensembl Genome Browser for the genes associated with intact L1 elements were recorded and tabulated. The expression of each gene in rat hippocampus was assessed using "Atlas Gene Expression Database" (http://www.ebi.ac.uk/gxa/).

Results

Presence of L1 specific transcripts in the hippocampus

The L1 retrotransposons are present in multiple copies in the rat genome and only a few copies are known to be transcriptionally active under stress conditions. We

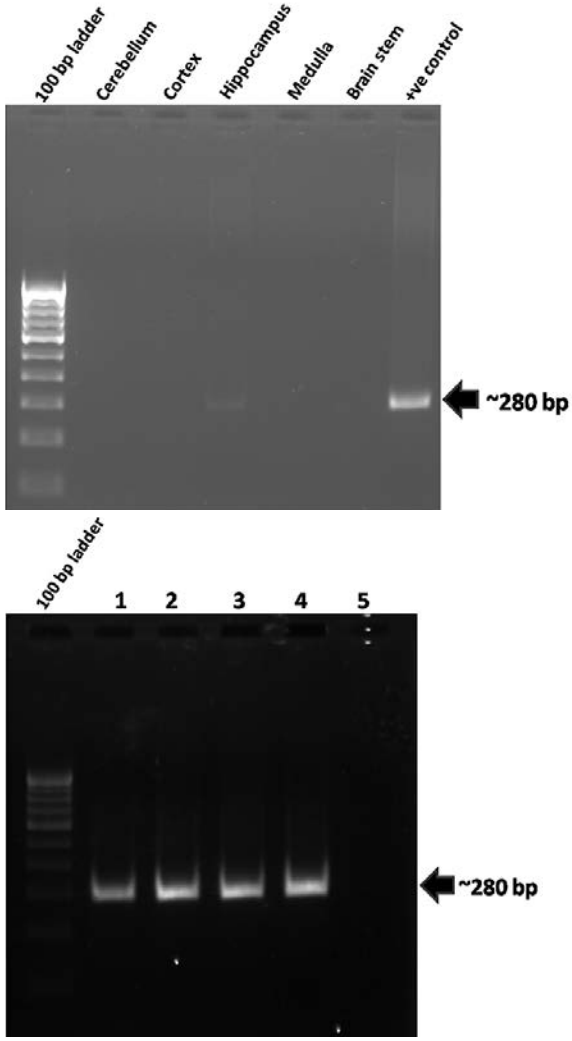


Figure 1 – RT-PCR profile of the L1 reverse transcriptase domain amplified using RNA isolated from different regions of brain. (A) RT-PCR amplification profiles of the L1 reverse transcriptase domain of different regions of brain: cerebellum, cortex, hippocampus, medulla oblongata, and pons. Approximately 280-bp amplicon (positive control) is expected using the specific primers. Amplification product is visible only in the hippocampus. (B) Lanes 1–4 – RT-PCR amplification of the L1 reverse transcriptase domain from the hippocampus of different rats. Lane 5 – negative control.

analysed the presence of L1 specific transcripts in various regions of rat brain under normal growth conditions. The degenerate primers corresponding to the L1-specific RTase domain are expected to produce an amplicon of ~280 bp. RNA isolated from five different regions (cerebral cortex, cerebellum, hippocampus, medulla oblongata and pons) of four-month-old rat brain were used for RT-PCR. The RT-PCR profiles show the presence of the L1-specific transcript only in the RNA isolated from the hippocampus region (Figure 1A). The results indicate that the L1 retrotransposons are transcriptionally active in the hippocampus region of rat brain. No L1-specific transcripts were observed in any other region of the brain. The experiments were repeated several times confirming that detectable transcriptional activity of L1 elements is only present in the hippocampus (Figure 1B).

Existence of different families of L1 transcripts

The reverse transcriptase domain of L1 retrotransposons is highly conserved at the amino acid level but not at the nucleotide level. Since a large number of different L1 retrotransposons are present in the genome, the kinetic complexity of the amplified transcript was analysed in order to identify transcriptionally active elements. Therefore, the amplified products were cloned and sequenced. Of the 37 clones sequenced, 20 were non-redundant sequences for L1-specific RTase domain suggesting that they possibly have originated from different L1 elements. All these sequences originating from transcriptionally active elements

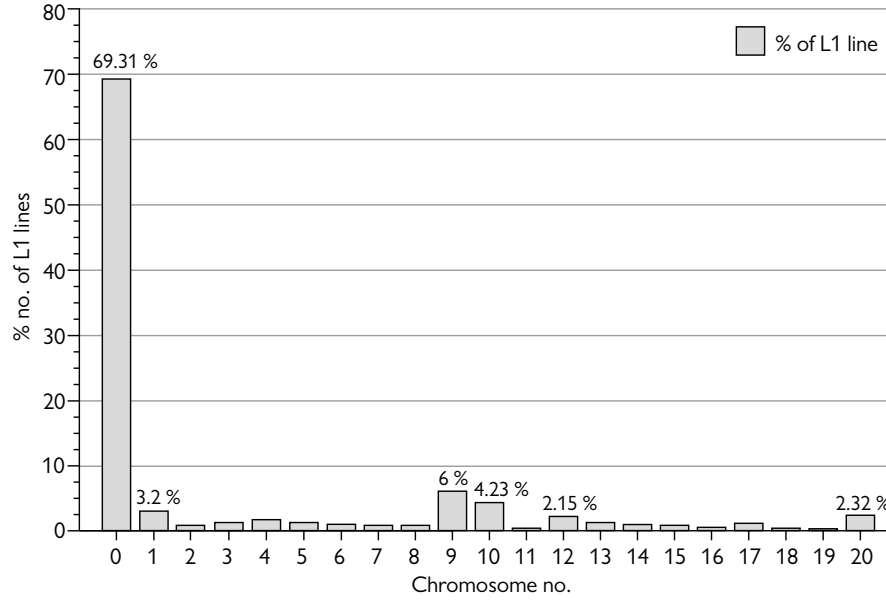
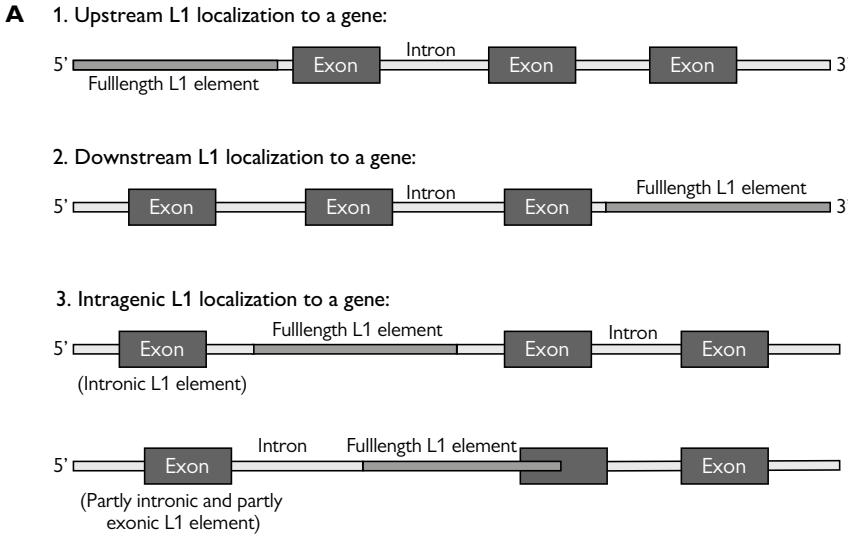


Figure 2 – Distribution of full length L1 elements in the individual chromosomes.

were analysed *in silico* to access the physical distribution of the hippocampus active L1 elements in the rat genome. Since the truncated elements could not be functional, only full length elements were taken into consideration. Analysis indicates a skewed distribution of the full length L1 elements on different rat chromosomes. Chromosome 1 contains over 69% of the full length elements and chromosomes 19 and 20 are almost devoid of any full length L1 elements (Figure 2). The chromosome 1 contains majority of full length elements but only a tiny fraction of these active in the hippocampus. The elements were considered active on the basis of sequence homology to the RT-transcript. And out of these



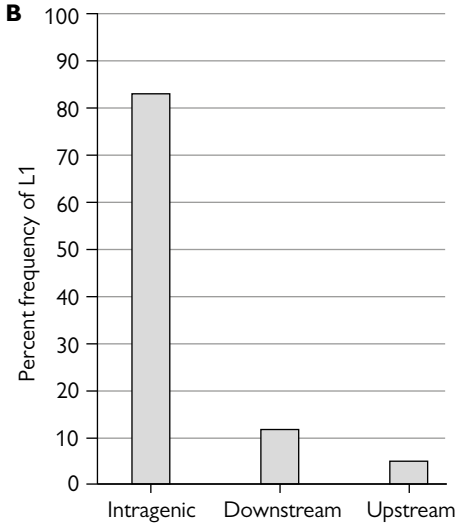


Figure 3A – Diagrammatic representation showing localization of full length L1 elements within or in the vicinity of the genes.
Figure 3B – Relative proportion of location of the L1 elements within or in the proximity to the genes.

a very few of the full length elements are located in the vicinity of the protein coding genes. Chromosomes 2, 4, 9, 13 and 14 contain 13, 9, 10, 10 and 7% of the transcriptionally active L1 elements respectively.

L1 elements are associated in close vicinity with genes

On searching of the L1 Rn database, we found 77 full length elements in the vicinity of genes. Of these full length elements, 63 are localized in the intragenic regions and the remaining ones were located upstream or downstream (within 10 kb) of the genes (Figure 3A and B). Figure 3B shows a relative distribution of the elements within or in the vicinity of the genes. At least 20 different members of the L1 family appear to be transcriptionally active in the hippocampus. The integration of a large proportion of these elements is within genes, either within an intron or in some cases also includes part of an exon. Functionality of the gene containing element within an intron may not be affected provided the integration does not affect the splicing.

Discussion

The rat genome contains 23% of the L1 family of retroelements and a proportion of the elements may potentially be mobile. They are known to be under tight regulatory controls and remain transcriptionally or transpositionally silent under normal conditions. They are known to be activated under environmental stress or metabolic perturbation (Muotri et al., 2009; Hunter et al., 2013; Kaer and Speek, 2013). We show that the retroelements that normally are transcriptionally/transpositionally activated in response to stress or metabolic perturbation could be active under normal physiological conditions in the hippocampus. Since the transcription of the elements is a prerequisite for retrotransposition, it is very likely that at least a part of L1-derived transcript would lead to retrotransposition. Besides retrotransposition activity has shown to be increased during embryogenesis and neurogenesis. Since the hippocampus is the primary site for neurogenesis it is possible that the enhanced transcriptional activity of L1 elements in the hippocampus may directly be related to neurogenesis.

Enhanced transpositional activation of L1 elements has been associated with high neuronal turn over leading to neuronal diversity and plasticity. In adult humans, 700 new neurons are added in the hippocampus every day. Substantial neurogenesis takes place throughout life in the human hippocampus except a modest decline during aging (Spalding et al., 2013). New neurons are continuously added and this high degree of neurogenesis continuously remodels hippocampal circuits (Akers et al., 2014). This remodelling possibly is a consequence of synthesis of new neurons replenishing the old ones. It is tempting to conjecture that the increased retrotransposition during neurogenesis may be related to the degradation of old neurons. However, there are contrasting observations with respect to increase in copy number of L1 elements in the hippocampus. Coufal et al. (2009) observed

an increased in copy number of L1 elements in the hippocampus and other regions of adult human brain as compared to the copy number of L1 elements in heart or liver. On the other hand, Evrony et al. (2012) analysed the genomic variability among the individual neurons of the brain by single neuron sequencing and concluded that no detectable level of L1 induced integration in neurons. They suggested that L1 was not a major generator of neuronal diversity.

The retroelements have mostly been considered evolutionary byproducts even though mammalian genomes contain a significant proportion of potentially mobile retrotransposons (Speek, 2001; Baillie et al., 2011). The transcriptional control on the elements is being exercised either through epigenetic modulations or by specific miRNAs (Macia et al., 2011). The regulatory controls are known to be relaxed during pathological conditions especially in cancer. Extensive transduction of non-repetitive DNA mediated by L1 retrotransposition in cancer genomes has been observed (Tubio et al., 2014). A significant proportion of protein coding genes and regulatory domains of the genes have been shown to be evolved from retrotransposons (Kokošar and Kordiš, 2013). The proximity of the active retroelements to protein coding genes may affect their expression. The L1 elements could be integrated within the gene in either orientation. The human and mouse L1 elements may have both upstream and downstream transcriptional capabilities due to presence of sense and anti-sense promoters at their 5' UTRs. Expression of genes such as *Nkain*3, *Mtif*2, *Atf*6, *Rabgap*1l, *Dock*1, *Lin7*a, *Cyp20*a1 etc. located in close proximity to the L1 elements may possibly be modulated by the active element in the hippocampus (Speek, 2001; Faulkner et al., 2009). Therefore, it is conjectured that limited transcriptional activation of elements may have specific role in neuronal differentiation.

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Juxtarenal Mycotic Aneurysm as a Complication of Acute Exacerbation of Chronic Cholecystitis Treated by Resection and Replacement by a Fresh Allograft

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Abstract: We present a case of a female patient with infectious (mycotic) juxtarenal abdominal aneurysm with atypical symptoms beginning as acute exacerbation of chronic cholecystitis. Apart from common antibiotic treatment, the patient successfully underwent resection of the diseased segment and replacement by a fresh allograft in order to reduce the risk of infection of the graft, but with the need of subsequent life-long immunosuppressive therapy. Perioperative monitoring of the spinal cord by near infrared spectroscopy was used to identify possible spinal ischemia. The choice of the fresh allograft was based on our experience supported by review of the literature.

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Introduction

In the recent years, we have observed increasing numbers of patients with infectious aneurysms with atypical disease course. We decided to report one particular case of juxtarenal mycotic aneurysm with atypical symptoms beginning as acute exacerbation of chronic cholecystitis. Apart from antibiotic treatment, the patient underwent resection of the aneurysm with replacement of the segment by a fresh aortic allograft in order to reduce the risk of infection of the graft. The procedure was performed in general anaesthesia with perioperative monitoring for spinal ischemia.

Case report

In September 2012, a 68-years-old female patient was admitted to a General University Hospital for abdominal and back pain. The pain was increasing gradually for a week, with maximum in the right upper quadrant and irradiation into the

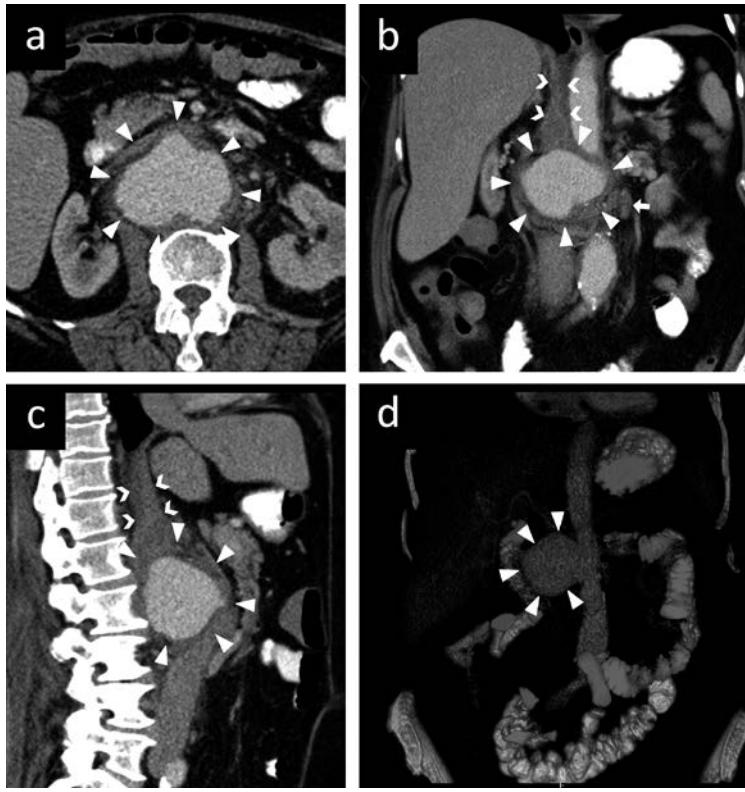

Figure 1a–d – CT of abdomen in portal venous phase shows large juxtarenal saccular mycotic aneurysm (72×51×66 mm, arrowheads) with thick (up to 7.4 mm) indistinct wall, stranding of adjacent retroperitoneal fat, enlarged lymph nodes (arrow) and signs of past leakage (chevrons) in axial (a), coronal (b), sagittal (c), and 3D (d) reconstructions.

right lumbar region. She reported no fever or chills. Initial clinical examination was unremarkable, but blood tests revealed elevated leukocyte count 14.1×10^9/l (reference = 4–10.7×10^9/l, Leu), and high C-reactive protein 220 mg/l (reference = 0–7 mg/l, CRP). Abdominal ultrasound showed solitary bile stone and signs of acute exacerbation of chronic cholecystitis (irregular thickening of the gallbladder wall up to 9.5 mm with increased echogenicity), liver steatosis, and aortic aneurysm 32×30 mm in diameter. Therefore, she was treated for acute exacerbation of chronic cholecystitis by cephalosporin antibiotics (cefoperazone, Cefobid®, Pfizer, Czech Republic, 2 g every 12 hours i.v.). However, this treatment had no influence on her symptoms and laboratory markers of inflammation were increasing (CRP 315 mg/l, Leu 16.7×10^9/l). Blood cultures were negative, but serology identified acute *Salmonella enteritis* infection. CT of abdomen was requested to rule out complications. It showed moderate thickening of the gallbladder wall outlined by a thin rim of fluid consistent with the previously stated diagnosis. More importantly, the juxtarenal aneurysm previously identified by ultrasound substantially increased in size (diameter = 72×51 mm, length = 66 mm) and became saccular in shape (Figure 1). Its wall was thick (up to 7.4 mm), indistinct, blurred with stranding of the adjacent retroperitoneal fat and signs of past leakage. One week after admission, the patient was referred to the Department of Cardiovascular Surgery for juxtarenal infectious (mycotic) aneurysm.

Because implantation of a stentgraft into an infectious aneurysm would pose a high risk of infection of the prosthetic material, we decided to perform replacement of the diseased segment by an allograft. The fresh allograft was available five days later.

Anaesthesia

Replacement of a 16 cm long visceral segment of the abdominal aorta was performed in general anaesthesia with selective intubation of the right bronchus and patient in the right lateral decubitus position. Prior to induction of anaesthesia, a thoracic epidural catheter was inserted at the level of T6-T7, as well as intraspinal catheter (Codman Neuro, USA) for drainage of the cerebrospinal fluid and measurement of the pressure inside the subarachnoid space. The anaesthesiologist applied complex hemodynamic monitoring including invasive blood pressure measurement above and below the aortic clamp during the procedure. Monitoring of advanced hemodynamic parameters, such as the stroke volume, cardiac output/index and systemic vascular resistance was achieved with a Vigileo system (Edwards Lifescience, USA) and heart performance was assessed with the transesophageal echocardiography (TEE) throughout the procedure. Regional dermatomal oxygenation at the level of L1 was continuously measured using near-infrared spectroscopic (NIRS) oximetry (INVOS, Covidien, USA) (Etz et al., 2013).

Surgical procedure

The aorta was accessed through a left thoracotomy in the fifth intercostal space (left lung was deflated) and midline laparotomy. There were inflammatory changes in the tissue around the aorta with enlarged periaortic lymph nodes. The aortic wall was of inferior quality with decreased compliance due to multiple atherosclerotic plaques. After introduction of a left heart bypass (from the left upper pulmonary vein to the left common femoral artery) under full heparinization (Heparin Leciva, Leciva, Czech Republic, 0.5 mg/kg), an aortic cross-clamp was placed on the thoracic aorta 5 cm above the diaphragm and on the abdominal aorta above the celiac artery. The proximal end-to-end anastomosis was constructed after transverse aortotomy 2 cm above the diaphragm. Then, a cross-clamp was placed onto the distal end of the allograft and on the aorta above the bifurcation. The diseased visceral aorta was resected while the origins of the celiac artery, upper mesenteric artery, and the left and right renal artery were connected to a biopump (BPX-80 BIO Pump Plus Centrifugal Blood Pump, Medtronic, USA) in order to maintain their perfusion. Then the visceral arteries were connected to the allograft in the following order: right renal artery (end-to-side), celiac artery (end-to-end), upper mesenteric artery (end-to-end), and left renal artery (end-to-end). Finally, the proximal aortic cross-clamp was removed to restore circulation in the visceral arteries and the distal aortic anastomosis was constructed (end-to-end). The biopump was suspended and after cancellation of heparinization by equal dose of protamine sulfate (Protamin ME, MEDA Pharma, Germany), control of bleeding, and placement of drains, the wounds were sutured.

Postoperative care

The double-lumen tube was replaced with a standard endotracheal tube at the end of surgical procedure. The patient was then transferred to a postoperative intensive care unit, ventilated, with circulation on a moderate vasopressor support (noradrenaline, 0.3 μg/kg/min). As hemodynamic parameters gradually improved, she was extubated on the third postoperative day.

On the 7th postoperative day, debridement of the laparotomy was performed because of purulent discharge (*Staphylococcus aureus* was identified by culture) and vacuum-assisted closure (VAC) system (V.A.C.® Therapy System, KCI, USA) was introduced and exchanged every four days, four times altogether. Later the wound was resutured.

The antibiotics were administered during the postoperative period for another four weeks. Postoperatively, we observed a marked decrease of CRP (23.6 mg/l) and Leu (7.15×10^9/l). The patient was discharged on the 25th postoperative day with the following medication: acetylsalicylic acid 100 mg in the morning (Anopyrin 100 mg, Zentiva, Czech Republic) as antiaggregant and tacrolimus 1 mg, 1 pill in the morning and evening, 2 pills at noon (Prograf 1 mg, Astellas Pharma, Czech

Republic) as immunosuppressant with dose adjustment to maintain plasmatic levels between 4 and 7 ng/l.

Follow up

One month after discharge, the patient did well and the operative wounds were healed. CT angiography showed 80% stenosis of the proximal part of the left renal artery (Figure 2), which was successfully treated by angioplasty and stenting two weeks later. Three months postoperatively, the patient underwent laparoscopic cholecystectomy. Histology showed chronic fibrous cholecystitis with foci of acute exacerbation.

Discussion

Infectious aneurysms represent 1% of all thoracic and 3% of abdominal aneurysms (Laohapensang et al., 2012). Patients with diabetes, immunodeficiency, drug users,

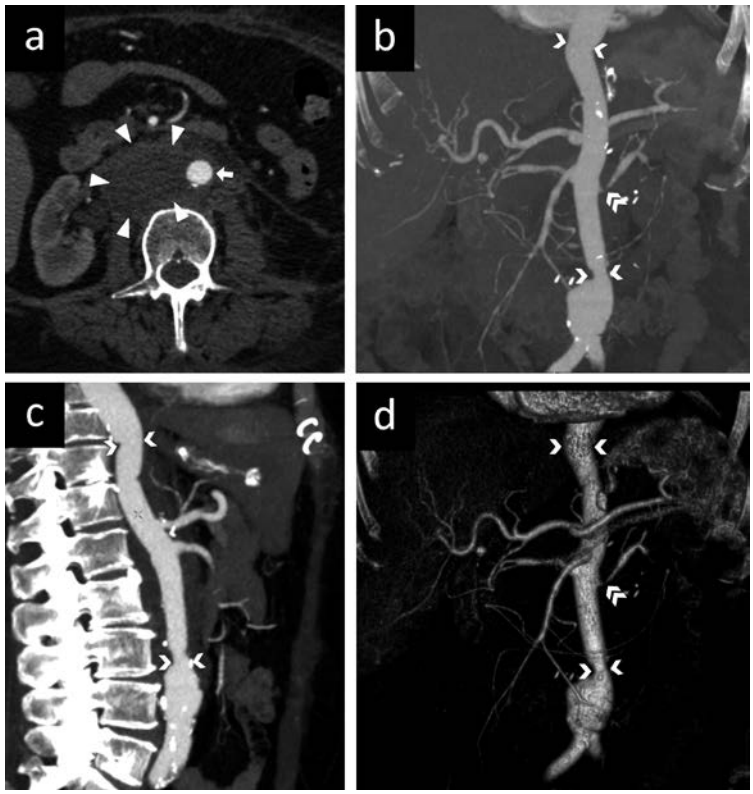

Figure 2a–d – CT angiography of abdominal aorta after resection of mycotic aneurysm and replacement by a fresh allograft shows good postoperative result apart from stenosis of the left renal artery (double chevron) and retroperitoneal hematoma (arrowheads) in axial (a), coronal (b), sagittal (c), and 3D (d) reconstructions. Chevrons indicate the proximal and distal anastomosis.

and patients after surgical procedures in the thorax (coronary bypass, replacement of the ascending aorta) are at increased risk (Karkos et al., 2014). The infection usually invades the aortic wall through a diseased intima (most commonly by atherosclerosis), or through vasa vasorum (Sharma et al., 2011). Apart from sepsis, patients with infectious aneurysms are endangered by imminent rupture of the aneurysm due to accelerated destruction of the aortic wall. Blood cultures are positive only in half of the patients. Even though all patients have elevated markers of inflammation (Leu, CRP, procalcitonin), the mainstay of diagnosis consists in cross-sectional imaging, CT, MRI, or PET-CT in particular (Steverlynck and Van de Walle, 2013).

The treatment of infectious aneurysms is complex. Its primary objective is to manage the infection by means of antibiotic therapy for at least six weeks. The definite treatment, however, is surgical resection of the aneurysm and bridging of the aortic segment by a prosthetic graft with the risk of its infection or by an extra-anatomic bypass, which has a limited durability. Pure endovascular treatment, first published by Semba et al. in 1998, that is reserved for high risk patients, who cannot undergo operation, has a high infection rate of the stentgraft (9–20%) with undiminished risk of subsequent rupture (Semba et al., 1998; Strahm et al., 2012). Therefore, replacement of the diseased segment by an allograft is a viable option. However, even this solution has its drawbacks: the allograft is usually not readily available and, above all, the patient has to be on lifelong immunosuppressive treatment. In the abdominal aorta, the reported mortality is lower, about 10%, but in the thoracic aorta prohibitively high, up to 45% (Scali et al., 2013).

Replacement of the abdominal aorta with reconstruction of the visceral arteries is a complex procedure and there are several technical aspects that need to be considered. Firstly, to maintain perfusion of the abdominal viscera, a biopump is the preferred option, because lower dose of heparin is required compared to other alternatives. Secondly, although the abdominal aorta can be accessed via the retroperitoneal approach, we chose the intraperitoneal access via the left paracolic gutter because it is less demanding, faster, offers better visualization of the operating field and direct assessment of perfusion changes of the abdominal viscera. Thirdly, hypothermia which may be desirable for protection of the spinal cord and the abdominal organs increases the risk of bleeding from the wound area, which is rather large in this procedure. Fourthly, although we prefer to perfuse the disconnected vessels with blood, which contains buffers and transports gases in physiological amounts, protective solutions such as Ringer's lactate solution with mannitol and solumedrol may be used alternatively. Fifthly, perioperative and postoperative monitoring of the spinal pressure is mandatory for early detection and management of hypertension that may be caused by edema of the spinal cord. Sixthly, antibiotic treatment was guided by the clinical and laboratory examinations and in the postoperative period it was terminated

after the fourth postoperative week based on mutual agreement with the Clinical Microbiology and Antibiotic Center, although the general recommendations suggest a minimum of 6 weeks.

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Snakebite Envenoming by Sochurek's Saw-scaled Viper *Echis Carinatus Sochureki*

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Key words: Snakebite – Saw-scaled viper – *Echis pyramidum sochureki* – Coagulopathy – Renal failure – Antivenom

Abstract: A snake breeder, 47-years-old man, was bitten by the saw-scaled viper (*Echis carinatus sochureki*). After admission to Toxinology Centre, within 1.5 h, laboratory evaluation showed clotting times prolonged to non-measurable values, afibrinogenaemia, significantly elevated D-dimers, haemolysis and myoglobin elevation. Currently unavailable antivenom was urgently imported and administered within 10 hours. In 24 hours, oligoanuric acute kidney injury (AKI) and mild acute respiratory distress syndrome (ARDS) developed. Despite administration of 10 vials of urgently imported Polyvalent Snake Antivenom Saudi Arabia, the venom-induced consumption coagulopathy (VICC) and AKI persisted. Another ten vials of antivenom were imported from abroad. VICC slowly subsided during the antivenom treatment and disappeared after administration of total 20 vials during 5 day period. No signs of haemorrhage were present during treatment. After resolving VICC, patient was transferred to Department of Nephrology for persisting AKI and requirement for haemodialysis. AKI completely resolved after 20 days. Despite rather timed administration of appropriate antivenom, VICC and AKI developed and the quantity of 20 vials was needed to cease acute symptoms of systemic envenoming. The course illustrates low immunogenicity of the venom haemocoagulation components and thus higher requirements of the antivenom in similar cases.

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Introduction

Saw-scaled or carpet vipers *Echis* sp. native in North Africa, Middle East and Central Asia (including India) are responsible for major part of snakebite morbidity and mortality in the places of occurrence (Warrell et al., 1977; Warrrell, 1995a, b). Sochurek's saw-scale viper *E. carinatus sochureki* (Figure 1) native in Arabian Peninsula, Iran, Afghanistan, Pakistan and India belongs to most venomous species of the genus. Saw-scale vipers are also rather popular among holders.

The main acting compounds of saw-scale venoms are toxic enzymes and toxins affecting haemocoagulation. Ecarin is one of the most clinically important components and it is direct activator of prothrombin to izoenzym meizothrombin, which is not inactivated by antithrombin-heparin complex and causes explosive thrombin activation (Gillissen et al., 1994; Lu et al., 2005). In contrary to disseminated intravascular coagulation (DIC), antithrombin, at least in the initial phase, remains intact or can be even elevated (Mba and Onyemelukwe, 1989). The uncontrolled thrombin generation produces fibrin formations, which are destructed by plasmin into large quantity of fibrin degradation products (FDP, D-dimer). Direct fibrino(geno)lytic enzymes also play some role in subsequent afibrinogenemia. The result is the consumption disorder – venom-induced consumption coagulopathy (VICC) (White, 2005; Brown et al., 2009).

Other venom components are desintegrins that inhibit the platelet (PLT) aggregation. Such activity can manifest inadequate participation of PLT in the consumptive coagulopathy (Warrell et al., 1977; Okuda et al., 2001). However, this does not eliminate the possibility of increased PLT agreggability by the other components and/or PLT consumption in fibrin formations.

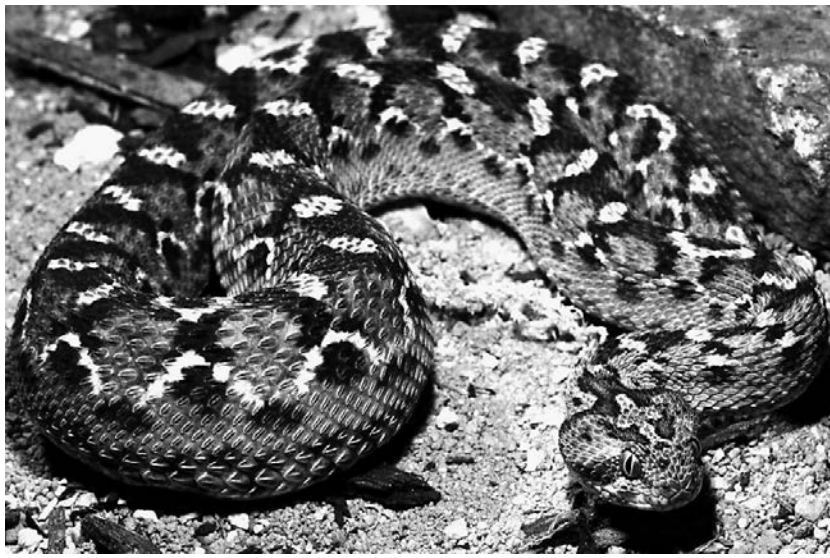

Figure 1 – Echis carinatus sochureki *(photo V. T. Jirousek).*

The venom of the *Echis* snakes, as the majority of vipers, contains haemorrhagins, which activate and disintegrate endothelium and its junctions, thus aggravating prothrombotic activation (White, 2005). In addition, there is a range of cytotoxic and destructive proteolytic enzymes causing local damage, including formation of necrosis (Annobil, 1993; Warrell, 1995a, b).

Clinical result of venom impact is then afibrinogenemia with possible haemorrhage and interstitial oedema with organ (i.e. respiratory) failure. Frequent AKI (acute kidney injury) results from multifactor action of above mentioned venom components (Merchant et al., 1989; Annobil, 1993; Top et al., 2006).

Case report

A snake breeder, 47-years-old man, was bitten into the thumb of left hand by the saw-scaled viper (*Echis pyramidum sochureki*). He attended a local hospital and subsequently was transported to Toxinology Centre of General University Hospital in Prague.

After admission, initial oedema of the thumb extended to the level of wrist. No other clinical signs of systemic envenoming were present. The laboratory examination, performed 1.5 hours after the bite, showed immeasurable values of clotting times, afibrinogenaemia, large quantity of fibrin degradation products, D-dimer, haemolysis, and myoglobin elevation up to 404 μg/l. Other laboratory results did not exceed the normal values. Count of platelets (PLT) decreased in 4 hours to 22.10^9/l (Table 1). Regarding these findings, which reflected serious systemic envenoming with venom-induced consumption coagulopathy (VICC), antivenom therapy was indicated. Because the adequate antivenom was unavailable at that moment, 10 vials of Polyvalent Snake Antivenom (Equine) Saudi Arabia was urgently ordered and transported from foreign Toxicology Centre. Fresh frozen plasma in quantity of 20 ml/kg was administered until antivenom became available to prevent development of potential serious haemorrhage. Simultaneously, crystalloid volume replacement therapy and furosemide IV was used for imminent acute kidney injury (AKI). Within 10 hours after the bite, three vials of antivenom were administered with no effect on coagulation parameters. Subsequently, another seven vials were used during that day. Following in total 10 vials of antivenom, the clotting times started to be measurable (Table 1). Despite this treatment, in 24 four hours oligoanuric AKI developed and ultrasound-guided central venous catheter for renal replacement therapy (RRT) had to be inserted. Continuous veno-venous haemodiafiltration (CVVHDF) with regional citrate anticoagulation was used. In the same time, mild respiratory failure with O_2 dependency developed. Chest X-ray showed a mild acute respiratory distress syndrome and echocardiographic examination excluded any cardiac failure. Ongoing hypoxemia required non-invasive ventilation support. In subsequent laboratory examination 24 hours after the bite, clotting times were found immeasurable again. Another ten vials of antivenom were ordered and the 2nd day five more vials were administered with positive effect in

Table 1 – Values of selected laboratory haemocoagulation parameters and antivenom administration

Day after bite	0	1					2			3	
Hours after bite	1.5	10	14	17	20	22	35	46	52		
Antivenom (vials)		3	2	2	3		3	2			
INR	>10	>10	>10	>10	>10	3.2	>10	3.23	2.09	2.39	1.40
APTT (s)	>180	>180	>180	>180	>180	149	>180	60.80	53.90	57.50	44.70
TT (s)	>180	>180	>180	>180	>180	93.7	>180	50.20	48.60	56.50	35.70
FBG (g/l)	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.10	0.19	0.12	0.21
AT (%)	97	81	74	70	64	63	77	66	68	68	76
D-dimer (μg/l)	>6400	>6400	>6400	>6400	>6400	>6400	>6400	>6400	>6400	>6400	>6400
PLT (10^9/l)	202	27	67	96	n/a	93	39	63	50	37	35

Day after bite	4		5		6	8	10	12
Hours after bite								
Antivenom (vials)		1	4					
INR	1.21	1.72	1.18	1.16	1.24	1.36	1.13	1.07
APTT (s)	40.40	43.00	33.30	33.20	31.70	37.70	33.30	28.60
TT (s)	46.50	54.90	30.30	31.10	25.40	19.90	17.10	16.60
FBG (g/l)	0.21	0.20	0.23	0.21	0.23	0.45	1.88	2.74
AT (%)	95	102	103	107	115	94	n/a	100
D-dimer (μg/l)	>6400	>6400	>6400	>6400	>6400	>6400	>6400	3031
PLT (10^9/l)	40	9	48	49	33	57	120	201

n/a – not available

shortening clotting times and slight elevation of FBG (fibrinogen) (Table 1). On day 4 and 5, dropped PLT count (pseudothrombocytopenia was excluded); no increase in FBG values and free haemoglobin > 700 mg/l signalized persisting influence of non-neutralised part of venom. Following this, another five vials of antivenom were used (Table 1).

The whole time of VICC lasting, no clinical signs of haemorrhage were present. Subsequently, the 6th to 7th day after the bite, the laboratory findings and clinical status normalised, except for D-dimer values and persisting AKI. The 10th day, the prophylactic mini-heparinization was initiated; uroinfection, confirmed in residual urine, was treated by antibiotics (Ciprofloxacin). The patient was transferred to Department of Nephrology at the 12th day for persisting AKI and the need of haemodialysis. AKI resolved in 20 days.

Discussion

The serious haemocoagulation disorder presents one of the most difficult problems for treatment. As VICC represents non-characteristic infliction of coagulation system, supplementary therapy like FBG or FFP (fresh frozen plasma) application does not improve the scenario, until the acting venom components are neutralised (White, 2005). However, in a case, when formed afibrinogenaemia brings a risk of serious haemorrhage and antivenom is not immediately available, administration of FFP can be a way to reinforce, at least temporarily, the ability of blood clotting. The second reason for clotting factors substitution could be slow onset of clotting equilibration after the venom neutralisation (Brown et al., 2009), particularly in higher risk of haemorrhage from other reasons, e.g. trauma, gastric ulcer, hepatopathy and others. This case demonstrates, that afibrinogenaemia lasting 48 hours, may not bring any haemorrhage. On that fact, substitution of FFP can participate as well as early antivenom treatment, even if it was titrated to laboratory findings and the time of neutralization was prolonged.

Another problem of VICC treatment is irregular antigenicity of haemocoagulation components, thus occasionally a need high and/or repeated doses of antivenom to neutralise this venom impact (Weis et al., 1991). That increases the time required to full restitution, when the total antivenom dose is titrated by haemocoagulation examination findings. The second approach to antivenom treatment is primary administration of high number of antivenom vials; but the total need of antivenom is not easy to estimate and high dosage of antivenom brings the risk of complications, e.g. serum sickness.

The use of heparin during the venom impact is controversial and not generally recommended (White, 2005). Nevertheless, according to Paul et al. (2003), heparin bolus of 5,000 units and further application of 2,500 units every 8 hours, in parallel with antivenom administration, can reduce mortality of persons envenomed by the saw-scaled viper *Echis carinatus* and the Russell's viper *Daboia russelli* from 26% to 19%. However these results are not highly significant. Heparin does not terminate

ongoing VICC and increases the risk of serious haemorrhage. But, after resolving of acute VICC with persisting activation of haemocoagulation and endothelial damage, prophylactic mini-heparinisation can prevent further possibility of thrombotic complications.

In this case adequately diagnosed respiratory failure of ARDS-type shows possibility of developing pulmonary affection, which was already registered by authors three times in other cases (Valenta et al., 2014). Unfortunately, this type of venom induced disability is not sufficiently evidenced in literature. The reasons may be short, mild and transitory course of respiratory failure and usually unsatisfactory blood gases monitoring and chest X-ray examination.

AKI developed rapidly despite of early antivenom treatment and provided volume replacement and absence of most reliable reasons: significant myoglobinuria (low level of serum myoglobin), haemoglobinuria, haemorrhage and hypotension. Developing AKI clinically confirmed influence of venom compounds, which can significantly affect the functional renal tissue.

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Terlipressin Induced Severe Hyponatremia

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Key words: Terlipressin – Hyponatremia – Adverse drug reaction – Vasopressin receptor – Fluid balance

Abstract: Terlipressin is a vasopressin analogue used for its vasoconstrictor effect in the treatment of variceal bleeding. Despite its good safety profile compared to vasopressin, some adverse reactions may occur during its use – e.g. hyponatremia. We describe a case of a cirrhotic patient with active variceal bleeding treated during two separate hospitalizations with terlipressin. In both drug treatment periods, severe laboratory hyponatremia developed. After terlipressin discontinuation, mineral disbalance corrected rapidly. Positive dechallenge and rechallenge corresponding to the drug administration schedule confirms the causality between terlipressin administration and hyponatremia. Hyponatremia was preceded with substantial fluid retention in both episodes. In this case report we want to highlight the need for fluid balance monitoring immediately after first terlipressin dose, which may individually predict the patient risk for the development of hyponatremia as other risk factors have rather limited predictive value in real clinical settings.

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Introduction

Terlipressin (1-triglycil-8-lysine-vasopressin) is a synthetic analogue of natural hormone vasopressin with long half-life and relatively low incidence of adverse effects (Freeman et al., 1982). It is frequently used in the treatment of gastrointestinal bleeding in patients with oesophageal and gastric varices, since it is a potent vasoconstrictor in the splanchnic circulation (Garcia-Tsao et al., 2007). Hyponatremia is a known adverse effect with estimated incidence of 0.1 to 1% treated patients according to the originator's summary of product characteristics. However, some publications estimate the incidence of this adverse effect higher in the real life use of the drug. Escorsell et al. (2000) report four cases of hyponatremia per 105 patients treated with terlipressin (3.81%), while Feu et al. (1996) report five cases of hyponatremia per 80 patients treated with terlipressin (6.25%).

Case report

A 34-year-old woman (60 kg, 165 cm) presented to the Department of Internal Medicine with hematemesis. The patient was known to have been diagnosed with oesophageal varices, Mallory-Weiss syndrome, portal hypertension and liver cirrhosis (Child-Pugh B) – most probably of ethylic etiology. When admitting, her blood pressure was 90/55 mm Hg, heart rate 70 beats/min, respiratory rate 14 breaths/min, and blood oxygen level 99%. Her initial laboratory findings were as follows: glucose 7.80 mmol/l, sodium 134.00 mmol/l, potassium 4.32 mmol/l, chloride 98.00 mmol/l, urea 5.84 mmol/l, creatinine 66.00 μmol/l, total bilirubin 47.00 μmol/l, conjugated bilirubin 17.00 μmol/l, aspartate aminotransferase 46.47 IU/l, alanine aminotransferase 13.52 IU/l, alcaline phosphatase 162.94 IU/l, gamma-glutamyl transferase 377.65 IU/l, alpha amylase 40.00 IU/l, C-reactive protein 34.10 mg/l, prothrombin time 17.50 s (international normalized ratio 1.43). Acute gastroscopy confirmed bleeding varices in the oesophagus and in the cardia. Bleeding was stopped by ligation rings deployment, the patient was placed in the intensive care unit and terlipressin therapy was started (1 mg at 22 h). The next two days terlipressin was administered at a dose of 1 mg every 4 hours, followed 1 mg dose every 6 hours for the next two days, further reduced to 1 mg every 8 hours for the next day. Severe hyponatremia with rapid onset began to develop on day 2 of terlipressin dosing (Figure 1). Sodium levels returned to physiological range also relatively rapidly within 3 days after treatment discontinuation. While fluid intake was stable of approximately 4,000 ml per day, fluid output was 1,650–1,900 ml for the period of full terlipressin therapy, in the course of discontinuation it gradually increased and after complete discontinuation it reached 8,050 ml. Variceal bleeding was stopped and after internal condition adjusting, the patient was moved to a standard care unit, from where she was discharged after three days.

Fifteen months later, this female patient was hospitalized for the same diagnosis again. Her status was: Child-Pugh B, blood pressure 110/80 mm Hg, heart rate

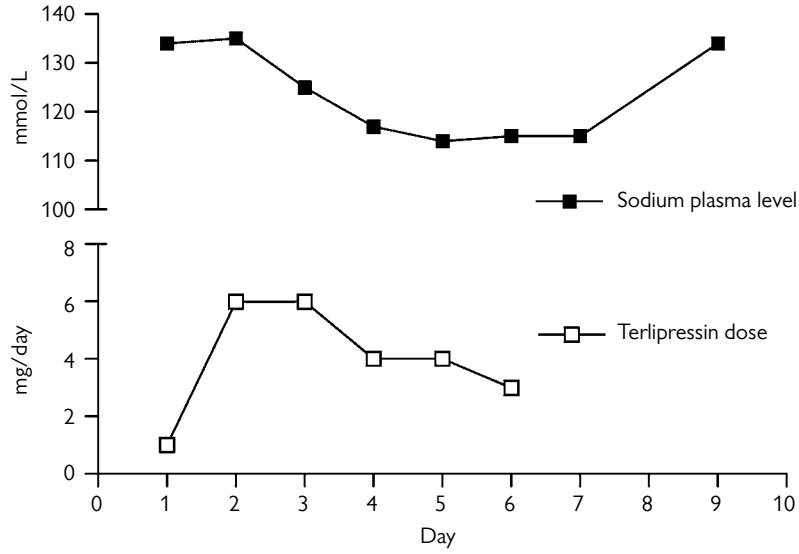

Figure 1 – Terlipressin dosing and plasma sodium levels during hospitalization.

110 beats/min, respiratory rate 14 breaths/min, and blood oxygen level 95%. Initial laboratory findings were: glucose 8.90 mmol/l, sodium 137.00 mmol/l, potassium 3.76 mmol/l, chloride 96.00 mmol/l, urea 8.04 mmol/l, creatinine 67.00 μmol/l, total bilirubin 69.00 μmol/l, conjugated bilirubin 44.00 μmol/l, aspartate aminotransferase 110.59 IU/l, alanine aminotransferase 22.94 IU/l, alcaline phosphatase 201.18 IU/l, gamma-glutamyl transferase 1325.88 IU/l, alpha amylase 21.76 IU/l, C-reactive protein 15.5 mg/l, prothrombin time 17.4 s (international normalized ratio 1.44). She was treated with terlipressin under the same schedule as in the first case. Severe hyponatremia developed rapidly again, however, minimum sodium level was numerically higher than in the first course of the treatment (126 mmol/l). Management of laboratory hyponatremia that was not accompanied with clinical symptoms in either of the two occasions included careful clinical monitoring and hypertonic saline administration.

Discussion

Three vasopressin receptor subtypes have been identified: V_1 (also known as V_{1a}), V_2 and V_3 (also known as V_{1b}). V_1 receptors are mainly located in smooth muscle cells of blood vessels, signal transduction is mediated by phospholipase C activation and release of intracellular calcium, and their activation leads to vasoconstriction. V_2 receptors are mainly located in renal collecting duct, signal transduction is mediated by adenylyl cyclase activation and increase in cAMP, and their activation leads to expression of aquaporins and their

incorporation into the membranes resulting in water reabsorption increase. V_3 receptors are mainly located in pituitary gland, signal transduction is mediated by adenylyl cyclase activation and increase in cAMP, and their activation leads to ACTH secretion increase (Saner et al., 2007; Krag et al., 2011). Terlipressin was developed as a selective V_1 receptor agonist. However, terlipressin has also substantial affinity to the V_2 receptors (Krag et al., 2008). As a result of V_2 receptors activation, water reabsorption in the renal collecting duct increases and dilutional hyponatremia may develop. This effect has been twice observed in our patient with diuresis reaching only less than half of the daily fluid intake during terlipressin administration and compensatory fluid overexcretion after terlipressin discontinuation. Since we observed severe hyponatremia on two separate treatment occasions with positive dechallenge (an adverse event which disappears on withdrawal of the medication) and rechallenge (symptoms re-occurring on re-administration) the causality between terlipressin administration and hyponatremia may be considered confirmed in our case. Kang et al. (2013) and Yim et al. (2015) identified that higher initial sodium plasma levels, lower patients' age, and preserved hepatic functions represent independent risk factors for development of hyponatremia. Our patient characteristics fit into this stratification, predicting the patients of increased risk for terlipressin-induced hyponatremia. However, the predictive value of these factors in reality is rather limited.

Of clinical importance is our observation that substantial fluid retention was noted within the first 24 hours after the first dose of terlipressin, while sodium level decline was first noted 32 hours after the first dose. Therefore for the practical terlipressin management, careful fluid balance monitoring immediately after the treatment initiation may be helpful for individual prediction of hyponatremia. Since the monitoring of fluid balance is a simple, non-invasive and very cheap technique not requiring any specific equipment or repeated blood samplings, it should be widely introduced into clinical monitoring of terlipressin treated patients for individual risk prediction.

Too rapid correction of serum sodium levels can cause serious neurological sequelae. Therefore plasma sodium correction must be realized carefully with frequent monitoring (Sterns, 2015). If the hyponatremia develops in the terlipressin therapy, it is also possible to consider the gradual discontinuation of drug, as in the described case reports.

Conclusion

Even though terlipressin-induced hyponatremia is a rare adverse reaction, it should always be taken into account when using this drug. Monitoring of fluid balance/retention shortly after the first dose of the treatment may be predictive for development of severe hyponatremia and should be introduced into the clinical terlipressin management monitoring scheme.

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