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Pancreatic Cancer Diagnostics and Treatment – Current State

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Abstract: Pancreatic ductal adenocarcinoma (PDAC) represents permanent and ever rising issue worldwide. Five-year survival does not exceed 3 to 6%, i.e. the worst result among solid tumours. The article evaluates the current state of PDAC diagnostics and treatment specifying also development and trends. Percentage of non-resectable tumours due to locally advanced or metastatic condition varies 60–80%, mostly over 80%. Survival with non-resectable PDAC is 4 to 8 months (median 3.5). In contrast R0 resection shows the survival 18–27 months. Laboratory and imaging screening methods are not indicated on large scale. Risk factors are smoking, alcohol abuse, chronic pancreatitis, diabetes mellitus. Genetic background in most PDAC has not been detected yet. Some genes connected with high risk of PDAC (e.g. BRCA2, PALB2) have been identified as significant and highly penetrative, but link between PDAC and these genes can be seen only in 10–20%. This article surveys perspective oncogenes, tumour suppressor genes, microRNA. Albeit CT is still favoured over other imaging methods, involvement of NMR rises. Surgery prefers the "vessel first" approach, which proves to be justified especially in R0 resection. According to EBM immunotherapy same as radiotherapy are not significant in PDAC treatment. Chemotherapy shows limited importance in conversion treatment of locally advanced or borderline tumours or in case of

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metastatic spread. Unified procedures cannot be defined due to inhomogenous arrays. Surgical resection is the only chance for curative treatment of PDAC and depends mainly on timely indication for surgery and quality of multidisciplinary team in a high-volume centre.

Introduction

Pancreatic cancer represents a permanent and ever rising issue worldwide (www.svod.cz; American Cancer Society, 2013). Nearly in 95% we deal with pancreatic ductal adenocarcinoma. The remaining 5% include acinar cells carcinoma, pancreatic blastoma and certain forms of cystic tumours (American Cancer Society, 2013). PDAC (pancreatic ductal adenocarcinoma) is still considered as the life threatening diagnosis, and despite enormous costs spent, specialists endeavour demonstrated, there virtually exists no effective treatment (Reznik et al., 2014). Statistically PDAC five-year survival rate does not exceed 3 to 6%, which is the worst result among solid tumours (American Cancer Society, 2013; Narayanan, 2015). Since 1977, the incidence of this highly aggressive carcinoma in the Czech Republic (CR) doubled (www.svod.cz) (Figure 1). In the United States, a total of 46 420 patients were diagnosed with PDAC in 2014, and 39 950 patients died of this illness during the same period (Becker et al., 2014; Edderkaoui and Eibl, 2014; Narayanan, 2015). It is expected that by the end of 2020 the number of PDAC cases will double up (Narayanan, 2015). Seriousness of this issue can be seen not only in the fact that the incidence is ever closer to prevalence, but in several other factors. In the USA, PDAC represents the fourth most frequent death causing tumour (7%), similarly to other western countries (placing between fourth and tenth most frequent),

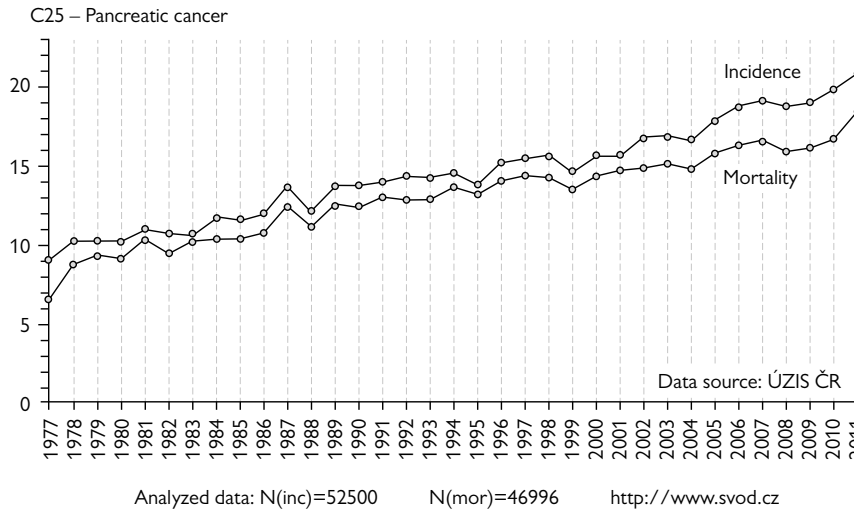

Figure 1 – PDAC incidence in the Czech Republic (array of 100 000 inhabitants, incidence – upper curve, mortality – lower curve).

albeit it represents only 3% of newly diagnosed tumours. Among gastrointestinal malignancies PDAC represents the second most frequent cause of death (www.svod.cz; Siegel et al., 2011; American Cancer Society, 2013; Becker et al., 2014; Edderkaoui and Eibl, 2014; Reznik et al., 2014; Narayanan, 2015). It remains one of the leading causes of cancer-related deaths worldwide, reflected by an incidence of 277 668 new cases and almost the same mortality rate (266 029 cases) per year (Siegel et al., 2011).

A principal difference (considering the chance of survival, yet relative to its length) can be seen, if diagnosis followed by surgical treatment is set in good time, i.e. the patient still benefits from the resection. Percentage of non-resectable tumours due to locally advanced or metastatic condition varies according to the literature from 60 to 96%, mostly over 80% (Lynch et al., 1990; Hoimes et al., 2009; American Cancer Society, 2013; Becker et al., 2014). Survival rate with non-resectable PDAC reaches 4 to 8 months (median value 3.5 months).

Hereditary component can be identified approximately in 10% of cases – familial PDAC, the rest is classified as non-familial sporadic form. Familial aggregation in patients with suspicion of hereditary genetic component was described already in 1973 (MacDermott and Kramer, 1973; Hoimes et al., 2009; Permuth-Wey and Egan, 2009; American Cancer Society, 2013; Canto et al., 2013; Conroy et al., 2013). In 1990 Prof. T. Lynch realized the first systematic study involving 18 families with PDAC and confirmed higher risk of its formation (Lynch et al., 1990; American Cancer Society, 2013). Since that moment, the systematic research focuses on this issue (Edderkaoui and Eibl, 2014).

Subject matter

PDAC diagnostics

Provided that the early diagnosis of potentially curative, or rather resectable pancreatic neoplasias, appears to be the only chance for life prolongation, the potential PDAC screening is the logical choice. Nonetheless, due to low PDAC incidence within the population and screening complexity, this method has not been widely recommended so far (Canto et al., 2013). Another reason is because there is no category of individuals within the population defined as a high-risk group (Hruban et al., 2010), except for the familial PDAC cases.

A further intense research aiming at detection and identification particularly among pre-cancerous lesions and especially at the cellular level, might improve screening efficiency. More precise and advanced endoscopic methods, as well as improved imaging of retroperitoneal region, also support the early diagnosis (Kolodecik et al., 2014).

Among risk factors of PDAC formation are not only numerous genetic syndromes, but also modifiable risk factor. Those factors together can increase the PDAC risk up to 132 times (Hoimes et al., 2009; Kolodecik et al., 2014).

Table 1 – Risk factors and PDAC (Becker et al., 2014)

Risk factor	Increased PDAC risk
Current cigarette use	1.7–2.2
Current pipe or cigar use	1.5
> 3 alcoholic drinks per day	1.2–1.4
Chronic pancreatitis	13.3
BMI > 40 kg/m^2, male	1.5
BMI > 40 kg/m^2, female	2.8
Diabetes mellitus, type 1	2.0
Diabetes mellitus, type 2	1.8
Cholecystectomy	1.2
Gastrectomy	1.5
Helicobacter pylori infection	1.4

PDAC – pancreatic ductal adenocarcinomas; BMI – body mass index

Established risk factors include a family history of pancreatic cancer, a medical history of hereditary pancreatitis, diabetes type II and cigarette smoking (Pelzer et al., 2013).

PDAC environmental risks, which involve smoking, diabetes mellitus, obesity, and alcoholism (Table 1) play considerably bigger role in the formation of this tumour than recognized, albeit ever rising number of studies focus on this topic (Go et al., 2005; Canto et al., 2013; Edderkaoui and Eibl, 2014; Kolodecik et al., 2014).

Influence of those elements is a matter of primary prevention.

Other studies in contrast deal with influence of blood types, where negative "impact" has been shown in type A (Pelzer et al., 2013, 2014). Chronic pancreatitis (CP) over a long period has been considered as a significant risk factor of PDAC. Meta-analyses document a relative risk of 13.3% of PDAC formation (Kolodecik et al., 2014). Chronic inflammation connected with CP can induce its progression into a tumour, and also cause development of three pre-cancerous lesion types:

- pancreatic intraepithelial neoplasia (PanIN)
- intraductal papillary mucinous neoplasm (IPMN)
- mucinous cystic neoplasm (MCN).

PanIN are microscopic ductal lesions. Most frequently recognizes as preneoplasic lesions seen in up to 82% of PDAC patients, and also in 16–80% of normal pancreas, albeit late lesions appear exclusively in PDAC patients. PanIN are mostly smaller than 1 cm and usually localized in the head of pancreas. In these cases, the possibility of detection by imaging methods is illusive. Their diagnostic detection is rather accidental or in a section.

The most serious in the three-grade classification is the PanIN 3 – carcinoma *in situ*.

IPMN accounts for 3–5% of pancreatic tumours and their classification derive from the degree of dysplasia. Invasive carcinoma is found in 20–50% IPMN. These

Table 2 – Selected PDAC genetic risk factors (Becker et al., 2014)

Risk factor	Gene	Increases PDAC risk	Other associated cancers
Hereditary breast and ovarian cancer syndrome	BRCA1, BRCA2, PALB2	2–3.5	breast, ovarian, prostate
Lynch syndrome (hereditary non-polyposis colorectal cancer)	MLH1, MSH2, MSH6, PMS2, EPCAM	8.6	colon, endometrium, ovary, stomach, small intestine, urinary tract, brain, cutaneous sebaceous glands
Familial adenomatous polyposis	APC	4.5–6	colon, desmoids, duodenum, thyroid, brain, ampullary, hepatoblastoma
Peutz-Jeghers syndrome	STK11/LKB1	132	esophagus, stomach, small intestine, colon lung, breast, uterus, ovary
Familial atypical multiple mole melanoma pancreatic carcinoma syndrome	P16INK4A/, CDKN2A	47	melanoma
Hereditary pancreatitis	PRSS1, SPINK1	69	
Cystic fibrosis	CFTR	3.5	
Ataxia-telangiectasia	ATM	increased	leukemia, lymphoma
Non-O blood group		1.3	
Familial pancreatic cancer	unknown	9 (1FDR) 32 (3FDRs)	

tumours may occur in the main or in secondary pancreatic outlets; if located in the main outlet, the risk of malignization rises up to 70%.

MCN are less common but relatively bigger tumours (1 to 3 cm), more often seen in females, with greater risk of malignization in 20% (Hruban et al., 2010; Kolodecik et al., 2014). Their development into a malignant tumour depends on several molecular changes. Despite the significant risk factor pancreatic carcinoma develops only in circa 5% of patients (Kolodecik et al., 2014).

About 10% of PDAC has a hereditary component, which complies with the familial incidence, i.e. one affected increases the risk of PDAC in the family by 80% (Becker et al., 2014).

Specific mutations in genes relate to about 10% of PDAC with different penetration and risk degree for each mutation (Table 2; Becker et al., 2014).

T. Kolodecik and his team studied possible pathways of PDAC development. Pancreatitis starts with an initiating insult followed by changes in the cellular environment and premature digestive enzyme activation. Mutations of genes associated with trypsinogen activation/inactivation predispose the pancreas to

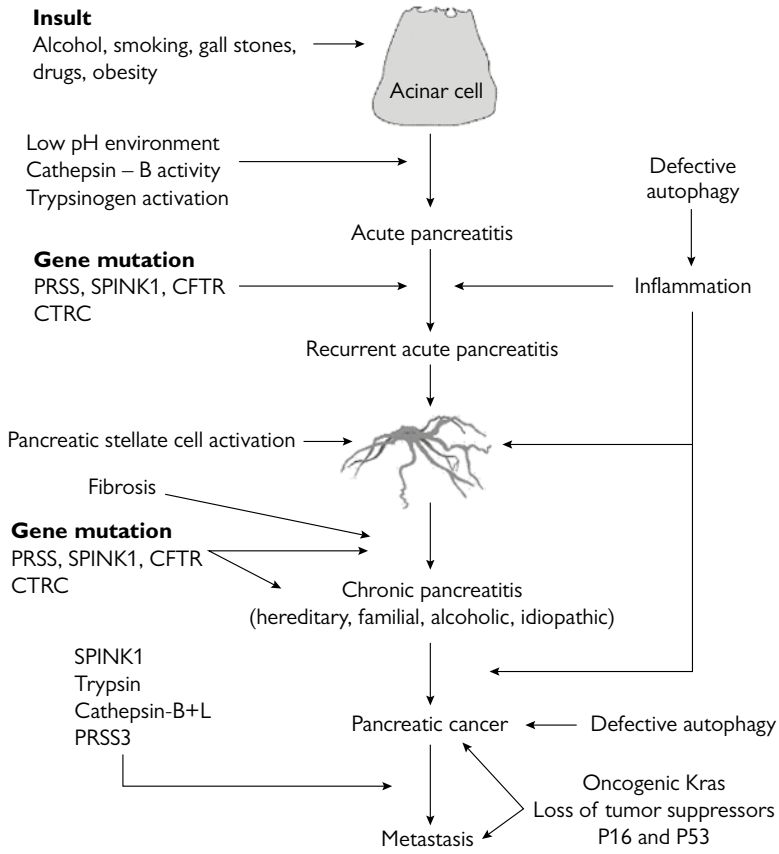

Figure 2 – Common pathways associated with disease progression from acute to chronic pancreatitis and pancreatic cancer.

development of disease. As disease progresses, defective autophagy, increased inflammation, pancreatic stellate cell activation, and fibrosis occur. Advancement toward pancreatic cancer and metastasis is also associated with defective autophagy, as well as extracellular matrix degradation, cell proliferation, expression of oncogenic KRAS and loss of tumour suppressors (e.g. P16 and P53) (Figure 2 – Kolodecik et al., 2014).

Number of studies dealing with gene mutations is on the increase (Conroy et al., 2013; Becker et al., 2014; Reznik et al., 2014). Their attention focuses mainly on DNA alteration of preneoplastic lesions.

Genetic cause in most PDAC has not been discovered so far. There have been some important and highly penetrative genes identified, such as BRCA2, PALB2, connected with the high risk of PDAC; however, the majority of PDAC cannot be explained by known genetic syndromes based on DNA familial testing. This finding

is reflected in the fact that only 10–20% of PDAC with familial aggregation indicate connection with highly penetrative genes. Remaining 80% need to be researched from the aspect of mutual links or by genetic sequencing.

Germinal mutation
Germinal mutation (germ-cell mutation) detectable in family members with PDAC embrace BRCA2 and some other e.g. FANCC and FANCG, PALB, PTEN, TP53, STK11/LKB1, p16CDKN2A, ATM, PRSS1 (influencing DNA in Fanconi anemia).

Somatic mutation
The genetic progression model for PDAC (compared for instance with adenocarcinoma development in CRC – colorectal cancer) represents sequential gain of proto-oncogene KRAS followed by mutation in tumour-suppressor genes, such as p16/CDKN2A/INKA4A, TP53 and SMAD4, that lead to disturbance of cell cycle regulation and initiate progression PanIN to PDAC. We assume that severe genetic mutation leading to sporadic PDAC are in fact mutations in proto-oncogene KRAS, as well as in tumour-suppressor genes p16/CDKN2A/INKA4A, TP53 and DPC4/SMAD4, while alteration in BRCA2, mismatch in repair genes (hMLH1, hMLSH2, and hMSH6), and AKT2 and STK11/LKB1 genes are rare.

Oncogenes
Oncogenesis in PDAC is supported by mutated and activated genes, particularly KRAS (located on 12p chromosome), BRAF (chromosome 7q), AKT2 (chromosome 19q) and AIB I (chromosome 20q). KRAS mutation is detected in up to 90% of PDAC, BRAF in 30%, and AKT2 amplification and overexpression in 10–60% of PDAC. Amplification AIB I in more than 60% of PDAC.

Tumour suppressor genes
These genes are recessive, and if inactivated they support tumour growth. Also, in PDAC a loss of important suppressor gene function can be seen; genes like p16INK4A/CDKN2A, TP53 and SMAD4/DPC4 are inactivated in more than 50% of all PDAC. SMAD4, located on chromosome 18q21, is inactive in about 50–60% PDAC. Also BRCA2 proved to be inactivated, but less frequently. Study focusing on other biomarkers also covers research of EGFG and VEGF expressions. Over the past few years, several studies focussed on assessment whether cytokine panel combination, specifically IL-6, IP-10, PDGF and CA19-9, can be utilised, same as biomarkers, for more precise PDAC diagnostics. According to some researchers, there might be some diagnostic potential (Hruban et al., 2000; Hoimes et al., 2009; Permuth-Wey and Egan, 2009; Delpu et al., 2011; Canto et al., 2013; Edderkaoui and Eibl, 2014; Krška, 2014b; Reznik et al., 2014; Narayanan, 2015). However, the CA (carbohydrate antigen) 19-9, also called sialylated Lewis blood group antigen, found in up to 95% of population in normal pancreatic ductal cells, still remains

the golden standard among laboratory diagnostics. Patients, who are Le^{a-b-} (Lewis blood group) negative do not evince any antigen expression even with large tumours. CA19-9 biomarker was described already in 1979 and still remains the only marker for PDAC diagnostics accepted by FDA (Federal Drug Agency) (Pelzer et al., 2014). CA19-9 serum levels in patients with chronic pancreatitis or benign biliary stricture are often elevated to about the same level as in small-scale PDAC. Another CA19-9 potential, apart from diagnostic importance, is seen in prediction of tumour recurrence after curative resection. Its sensitivity to PDAC varies between 71–81% and specificity between 83–90% at the cut-off level of 34.7 U/ml. The higher the cut-off level is (already 100 U/ml), the bigger the probability of recurrence, the lower the median of survival and percentage of five-year survival. The level is also influenced by high bilirubin or lack of fucosyltransferase (www.pathologyoutlines.com/pancreas.html; Delpu et al., 2011; Edderkaoui and Eibl, 2014; Krška, 2014b; Strobel and Büchler, 2014).

Another monitored marker ranks to microRNAs (miRNA). MicroRNAs are biologically stable and influence carcinogenesis. They are short non-coding RNAs composed of 18–25 nucleotides. They function to impact post-transcript regulation of gene expression leading to mRNA degradation, or possibly repression of mRNA translation, modifying cell proliferation, migration, and invasion and metastasizing.

In relation to PDAC more than 100 miRNAs have been identified. They can be assessed in aspirate, serum, bile or punctured sample. Assessment of suitable miRNA panel, mostly in concordance with other markers monitoring, is performed by many centres. The attention is focussed mainly on miRNA-10b, -155, -106b, -196a, 1290, and others (www.pathologyoutlines.com/pancreas.html; MacDermott and Kramer, 1973; Lynch et al., 1990; Hruban et al., 2000, 2010; Hoimes et al., 2009; Permuth-Wey and Egan, 2009; Delpu et al., 2011; Siegel et al., 2011; Canto et al., 2013; Conroy et al., 2013; Becker et al., 2014; Edderkaoui and Eibl, 2014; Krška, 2014b; Reznik et al., 2014; Narayanan, 2015).

The list of suitable biomarkers monitored for possible assessment of PDAC recurrence after resection is shown in Table 3.

Table 3 – Biomarkers evaluated for predicting recurrence following resection of PDAC (Osayi et al., 2014)

Carbohydrate antigen 19-9 (CA19-9)	Metastin
Carcinoembryonic antigen (CEA)	Phosphate and tensin (PTEN)
Cellular biomarkers	Molecular biomarkers
Circulating tumour cells (CTCs)	CX chemokine receptor 4 (CXCR4)
Neutrophil-lymphocyte ratio (NLR)	Cathepsin B
Gene biomarkers	Vascular endothelial growth factor (VEGF)
P16/CDKN2A, TP53, and SMAD4/DPC4	MicroRNAs (miRNAs)

Pathogenesis of PDAC has been most often correlated with the alterations of KRAS, P16, P53, DPC4 and FHIT (fragile histidina triad protein). Investigation of the alteration together with some of the miRNAs that are intensively investigated now appears as the most promising in this field of diagnosis.

Diagnostics – Imaging methods

Imaging methods play crucial role in PDAC diagnostics. Over the past three decades, some diagnostic methods, such as angiography and hypotonic duodenography, have been abandoned. Also ERCP (endoscopic retrograde cholangiopancreatography) is no longer perceived as necessary and is indicated only selectively. The golden standard in subhepatic region and retroperitoneum examination in case of PDAC suspicion is a multi-detection spiral CT (computed tomography). With each upgraded generation of imaging equipment, the sensitivity and specificity is ever more precise. Application of NMR (nuclear magnetic resonance) for mentioned indication is also wider for its ability to display outlet systems, which is beneficial. PET (positron emission tomography) CT is not applied as standard method in case of primary examination; its benefit is seen at the time of dispensarization (www.pathologyoutlines.com/pancreas.html; Hruban et al., 2000; Go et al., 2005; Delpu et al., 2011; Bockhorn et al., 2014; Diener et al., 2014; Edderkaoui and Eibl, 2014; Krška, 2014b; Osayi et al., 2014; Strobel and Büchler, 2014).

The basic algorithm of examination, which must be fast and as accurate as possible, it is therefore serological (markers as CA19-9) and practically parallel the CT examination rated according to protocol or endosonography with a biopsy. According to the experience of the workplace can CT replace NMR.

Tactics and extent of surgical procedure

Since the forties of the past century, the extent of surgical procedure has not changed significantly. Whipple procedure (pancreaticoduodenectomy) is a major surgical procedure performed if the tumour is located in pancreatic head, comprising resection of pancreatic head and duodenum. When the tumour is located in pancreatic body or tail, then this part is removed. Pancreatectomy is indicated in more developed or diffuse forms of PDAC. The extent of lymphadenectomy (LA) is based on D dissection, D2 or D3; LA does not bring any benefit. Pylorus preserving procedure (ppWhipple), so often referred to in scientific literature, brings almost no benefit in meta-analyses; on the contrary, in the short-term horizon, it represents higher risk of the upper type passage disorder; long-term results are still in the phase of research (Krška, 2014a).

However, overall perspective on the extent of procedure in case of vessel impairment has changed. If R0 (resection border without the presence of macroscopic and microscopic tumour involvement) resection can be expected, procedure on vessel system might be indicated (v. mesenteria superior, v. portae).

In case of arteries infiltration, indication for procedure on hepatic artery is a matter of consideration, yet in case of circulatory impairment of penetration into a. mesenterica superior, the procedure is hardly ever indicated. Some centres do perform this procedure also in the case of recurrence (minor localized recurrence and in a long-term distance from the primary operation), but only if it is reasonable (Bockhorn et al., 2014; Krška, 2014a; Strobel and Büchler, 2014).

Considering the procedure methods an important trend immerged, i.e. vessel first approach consisting in dissection of hepatoduodenal ligament structure, than complete loosening of duodenum and dissecting of the upper mesenteric artery at the point of its clearance from aorta. This technique enables not only to see the extent of tumour spread right in the initial phase, but also to loosen the tumour and surrounding tissue if necessary directly from the artery, i.e. the whole "meso-pancreas". This way any possible procedure on vessel structures or ligament becomes easier (Bockhorn et al., 2014; Strobel and Büchler, 2014).

Current ISGPS (International Study Group of Pancreatic Surgery) criteria for locally advanced tumour resectability are:

- maximum time-lapse from the last CT of 4 weeks;
- assessment by multi-disciplinary team in large-volume centre.

Technical criteria for possible resectability (i.e. not excluding surgical removal) are:

- constriction or closure of v. portae (VP), v. mesenterica (VMS) and their branching by tumour;
- penetration a. gastroduodenalis or a short part of a. hepatica into tumour, yet without impairment of truncus coeliacus;
- contact of tumour with mesenteric artery superior within circumference under 180 degrees (Hruban et al., 2000; Kelsen et al., 2008; Delpu et al., 2011; Bockhorn et al., 2014; Diener et al., 2014; Edderkaoui and Eibl, 2014; Krška, 2014a; Osayi et al., 2014).

Measures of indication to surgical exploration and resection in case of VP and VMS impairment are:

- evidence of resectability and possible vessel reconstruction;
- no evidence of neoadjuvant treatment;
- possibility of intra-operative decision for resection with vessel reconstruction (only if chance for complete tumour removal);
- type of vessel resection must be classifiable and describable.

General approach of surgeons to indication, exploration and possible resection in case of suspicion of arterial infiltration or occurrence:

- arterial reconstruction – not primary option (lack of evidence about benefits in pancreatic head impairment);
- recommended – surgical explorations should be performed to clarify arterial infiltrations observing the resectability border-line criteria (see above);

- fact that palliative treatment in case of arterial infiltration is the standard;
- concepts of neoadjuvant treatment and non-curative resections should be performed only in the scope of proper clinical trials.

Laparoscopic (LS) techniques are applied particularly in left-side resections or in tumours with possible enucleation. Introduction of staplers and modified transection methods rather increased the number of fistulas. LS procedures in the region of pancreatic head are possible and feasible, however due to complicated dissection with possible vessel involvement they are rarely indicated even in large centres also with respect to R0 resection necessity.

Laparoscopy indicated as the initial surgical step, for more than 20 years already, can confirm or exclude metastatic process, its benefit considering the resectability in locally advanced tumour is questionable. Considerable time parameters, especially for comorbid patient, are not suitable for LS procedures of pancreatic head.

During evaluation of pathological findings from surgically removed section the R-1 definition must be clear, i.e. tumoural cells in the section line (vs. 1 mm or more from the borderline), examination results of all seven evaluated lines, and thorough examination of the vessel wall (in case of its section).

To sum up, based on available data, the primary operation with vessel resection (in patients with locally advanced pancreatic cancer and borderline resectability) can be recommended, on the presumption that all necessary conditions are respected. These complicated procedures should be performed only in specialized high-volume centres with available erudite intensive care (Bockhorn et al., 2014; Diener et al., 2014; Krška et al., 2014; Strobel and Büchler, 2014).

Anastomosis complications, their evaluation and comparison are one "never-ending story". Considering that pancreatic surgery relates to 3–5% of peri-operative lethality and up to 40% of morbidity, the most serious complication, next to bleeding, is a pancreatic fistula. No existing method can eliminate occurrence of fistulas. The cause for this complication is multi-factorial; it depends on the condition of pancreas, the surgeon, "deep-rooted" operating skill, comorbidities, etc. Existence of this complication led to development and finding of other techniques (3-layer anastomosis, telescopic connection, outlet conversion to stomach, etc.).

Even preventive administration of somatostatin did not bring any change; only highly-developed, sophisticated operation technique of the large-volume centre can have some impact.

The strategy in metastatic PDAC is directed to adjuvant and symptomatic treatment. However, in many centres, an isolated metastasis in liver (in case of tumour resectability) is indicated to surgical section and procedure on pancreas (Diener et al., 2014; Hoskovec et al., 2014; Petruželka, 2014; Strobel and Büchler, 2014).

PDAC system treatment

Neoadjuvant oncological system treatment for resectable PDAC is not indicated. Benefits of this treatment have not been proved; the only chance for the patient is the surgical procedure R0 resection (Bockhorn et al., 2014; Diener et al., 2014; Strobel and Büchler, 2014).

Analysis of PDAC prevalence and incidence curves and their development very clearly shows only a slight effect of the system oncological care in other PDAC forms, despite diverse interpretation of "company trials". Results of individual trials documenting survival rate differ in weeks, and since the arrays are non-homogenous, meta-analyses can hardly be valid. The wish here is often father to the thought. The era of PDAC chemotherapy (CHT) began in 1996 by introduction of gemcitabine, rather expensive those days, and its global expansion. Results were slightly better than with 5-fluorouracil in the same indication. Before this era, oncologists generally had a very restrained approach towards this treatment.

Gradual development in this field brought combination of gemcitabine with erlotinib (from 2005), however gemcitabine treatment dominated. Situation changed with FOLFIRINOX combination, which suited patients with better performance status. Since 2012, combination of gemcitabine with nab-paclitaxel is used (Gunturu et al., 2013; Petruželka, 2014).

As for the conversion treatment of border-line resectable and locally advanced non-resectable tumour, many studies refer to application of FOLFIRINOX combination in preference to gemcitabine. The most optimistic studies state up to 40% possibility of conversion to resectable state and achievement of 20–30% R0 resection (Hosein et al., 2012).

Such "success rate" is frequently opposed by queries like: 1) primary staging often performed only by means of radiology methods; 2) differences between surgeons; 3) effect of chemotherapy (possibly also chemoradiotherapy) on inflammatory changes around tumour; and many others. It is a fact that conversion therapy must to be a part of oncosurgical team armamentarium.

Sorting of patients with metastasizing PDAC with regard to palliative therapy is described as follows (Petruželka, 2014):

1) Patients with good performance status – combined CHT FOLFIRINOX or Nab-paclitaxel/gemcitabine (10–32% patients);
2) Patients unsuitable for inclusion in array No. 1 – combination of gemcitabine with oxaliplatine or fluoropyrimidines; or gemcitabine with erlotinib (20–30% patients);
3) Patients with poorer performance status, comorbid and biologically older patients – mono-therapy by gemcitabine (20–30% patients);
4) Patients on supportive treatment without system therapy (5–30% patients).

Similarly to chemotherapy, radiotherapy apparently also has certain effect to QoL (decrease of tumour and lower pressure to retropancreatic nerve plexus). PDAC radiotherapy (RT) for conversion and adjuvant treatment is widespread

within Asian and American methods and trials. The convention as well as proton radiation, which by the way seems to be theoretically the most promising right now, bring temporary reduction of local impairment. However, there is only a minor influence to possible recurrence or generalization – the crucial cause of lethality.

Generally we can state, that neoadjuvant regimes, radiation and immunotherapy do not play any major role in clinical practice arising from "evidence based medicine" (Strimpakos et al., 2010; Lee et al., 2012).

Locally advanced PDAC – Ablation techniques

Over the several recent decades, some new forms of treatment methods were established (radiofrequence ablation, stereotactic body radiation therapy, high-intensity focused ultrasound, iodine-125-cryosurgery, photodynamic therapy, microwave ablation, irreversible electroporation, and others). Though their application is possible and from the technical aspect relatively easy, there is quite significant risk of serious complications (inflammation, bleeding, and fistulas). They can influence the local impairment, same as radiation therapy, yet the total survival rate remains unaffected. However, by local improvement, achieved for instance by IRE (irreversible electroporation), QoL improves as well (Lee et al., 2012; Boone et al., 2013; Hoskovec et al., 2014; Krška et al., 2014; Pelzer et al., 2014).

Conclusion

The only treatment technique, which in fact can influence not only the survival parameters, but also QoL, remains the R0 surgical resection. All other curative methods are only coarsely palliative and prolong the survival time just temporarily at the most. The principle factor here is timely recognition and indication to surgery. Utilization of diagnostic markers is still subjected to intensive research and we can see huge progress, however unequivocal results unfortunately are not available so far. Radiodiagnostics continues to be one of the principal methods. Primary CT results (not revision ones) should not exceed one month at the time of operation, which unfortunately is still the case in most centres. Main differences between centres can be seen in five-year survival rates and closely relate to arrays of patients and quality of the primary care. High-volume complex centres and highly experienced oncosurgical teams achieve better results. Trophy operations or non-indicated trials in PDAC are highly *non lege artis*. Further research will be directed to high-risk population groups with focus on familial and hereditary detection, onco- and tumour suppressor genes monitoring, and excreted cell parts (sudden formation of diabetes mellitus (DM) type 2, familial occurrence, chronic pancreatitis, and others).

Dedicated to Professor Marie Pešková, DSc. (1935–2008) the significant representative of pancreatic surgery at the occasion of her nearly 80 years birthday anniversary.

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Changes in the Concentrations of Corticoid Metabolites – The Effect of Stress, Diet and Analytical Method

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Key words: Cortisol – Cortisone – Aldosterone – Corticosterone – Stress – Food intake

Abstract: A precisely measured corticoid level is important for decision-making in daily clinical practice. These levels can be influenced in the pre-analytical phase, when the effect of stress, timing, and diet can be important. The aim of this study was to elucidate optimal conditions for blood sampling as well as the choice of analytical methods, which they will be used in measuring of corticoids. By studying ten women, we focused on the influences of the stress of cannulation and a large lunchtime meal on cortisol, cortisone, aldosterone and corticosterone levels. We further compared results of cortisol measurements from RIA and LC-MS/MS. Stress from cannulation caused increase of cortisol, cortisone and corticosterone already, when the cannula was being inserted. This indicates that this increase is stimulated by fear of the blood withdrawing rather, than just by the needle insertion itself. The effect of stress on corticosterone disappeared after an hour, while effect on other corticoids was still apparent. Concerning the lunchtime meal, we found an increase in all measured corticoids between 11 and 12 o'clock. After the food, there were marked decreases in cortisone and aldosterone, while declining levels of cortisol and corticosterone had rather plateaus. We compared cortisol in 90 plasma samples measured by a commercial RIA kit and the LC-MS/MS method. Results from both methods showed a strong correlation (r=0.85). When measuring corticoid metabolites, the chosen analytical method, eliminating stress factors, and precisely timed blood sampling considering the daily rhythm and food intake are critical.

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Introduction

Measuring plasma cortisol levels is a common task of clinical biochemistry laboratories, and not just when ordered by endocrinologists. However, in the pre-analytical phase there are various influences that can distort the information sought. These influences include stress brought about by the sampling, poorly-timed sampling, not taking the sampling after fasting, and choosing a method that is incompatible with the desired aims. Errors in the pre-analytical phase significantly complicate the interpretation of results, and could lead to an incorrect diagnosis.

Cortisol has a marked circadian rhythm in secretion that consists of a decline before bedtime followed by a maximum decline at midnight to under 100 nmol/l, and then an increase starting two hours before waking. Later during the day cortisol again declines and the cycle repeats. The basis of cortisol circadian rhythm is formed by episodic pulses occurring with almost hourly frequency – an ultradian rhythm. Changes to cortisol levels as part of the circadian rhythm are based on changes in the amplitude of these ultradian pulses (Lightman and Conway-Campbell, 2010).

There is a significant rise in cortisol in the first hour after waking, and these higher levels are the basis for the cortisol awakening response (CAR). Both serum and plasma cortisol levels increase by about 50–70% during the first 30 minutes after waking and remain increased for about 60 minutes (Pruessner et al., 1997). For this reason, if not specifically designed to analyse CAR then sampling for measuring cortisol levels should be performed at least one hour after waking.

In addition to the morning rise in cortisol, slight increases in cortisol levels associated with food have been described. We have performed two studies focused on the influence of daily timing and food intake on steroid hormone levels. In the first study we mapped changes in steroid hormones in relation to the time of day and regular food intake over 16 hours. We found decrease of cortisol levels after the main meals, which was probably due to withdrawals hours apart from meals (Rácz et al., 2015a), so we made a second more detailed study.

We tested this influence of food intake on cortisol levels in a second study, analysing levels after various forms of stimulation. In addition to a standard breakfast we compared levels after oral (OGTT) and intravenous (IVGTT) glucose tests as well as after ingesting psyllium as a model of the mechanical stimulation of the gastrointestinal tract (Rácz et al., 2015b). Using identical analytical conditions, we found differences in the trends in cortisol levels after these different food stimuli, but levels after 120 minutes were all the same. There was a decrease in the physiological decline of cortisol levels after each of the stimuli except psyllium. This decrease was most marked after IVGTT, lasting up to 60 minutes. After OGTT and IVGTT there was also a plateau in cortisol levels. After the standard breakfast there was an increase in cortisol levels after 40 minutes (Rácz et al., 2015b).

The aim of this present study was to expand on those findings in order to determine the optimal conditions for cortisol sampling as well as the choice of

analytical method. Firstly, we analysed the influence of stress due to cannulation on cortisol levels. Next, we focused on a detailed mapping of changes around the time of the main meal of the day, which in our country is lunchtime. Then, we compared measurements of cortisol using two analytical methods, radioimmunoassay (RIA) and liquid chromatography with tandem mass spectrometry (LC-MS/MS).

The newly-developed LC-MS/MS method allows multiple steroids to be measured at one time. We therefore expanded the study to analyse not just cortisol but all main corticoids after stress brought on by cannulation. We measured the main glucocorticoids, cortisol and its metabolite cortisone, plus the main mineralocorticoids, aldosterone and its precursor corticosterone, which is a minor steroid in humans.

Material and Methods

The study was performed using 10 healthy women of reproductive age in the follicular phase of their cycle (days 1–7 after menstruation). The average age was 33.6 ± 2.56 years and average BMI 25.06 ± 1.3. The women had no chronic diseases, were non-smokers, and did not use hormonal contraceptives or any other medications. Before starting the study, they were advised to maintain a balanced regimen of 8 hours of sleep, regular eating according to a recommended menu, and restrain from consuming alcohol. All participants were given explanations about the study and signed informed consent. The study was approved by the ethical commission of the Institute of Endocrinology.

Each woman passed two tests during two consecutive menstrual cycles:

1) Stimulation test by stress and food

Each participant woke up at 6:30 in the morning. They had at 7:00 standard breakfast (two slices of bread, 50 g of breast-meat chicken slices, 1 slice of fresh cheese; total caloric content of the breakfast was 515 kcal, total protein content: 20.58 g, total carbohydrates: 47.75 g, total fat: 24.9 g). The test started at 10:00, when cannula was inserted into the forearm or cubital vein. The time schedule – according to which the blood was drawn – was: 10:00, 10:15, 10:30, 10:45 and 11:00.

After the blood sampling at 12 o'clock, the participants of this part of the study received lunch (beef broth soup, turkey, potato dumplings, and sauerkraut; total content of the lunch was 679 kcal, total protein content: 45.55 g, total carbohydrates: 100.4 g, total fat: 11.5 g). The lunch was followed by blood drawings at 12:30, 13:00 and 13:30.

2) Blank test

Each participant woke up at 6:30 in the morning. They had at 7:00 standard breakfast (two slices of bread, 50 g of breast-meat chicken slices, 1 slice of fresh cheese; total caloric content of the breakfast was 515 kcal, total protein content:

20.58 g, total carbohydrates: 47.75 g, total fat: 24.9 g). The peripheral cannula was inserted at 7:30 into the forearm or cubital vein. The blood was drawn according to the same schedule as it was in the first part of the study, it means, that at 10:00 was drawn the first blood sample, which was followed by additional samples at 10:15, 10:30, 10:45 and 11:00. In contrast to the stimulation test, in the blank test participants did not have any lunch at 12 o'clock, but they were submitted to samplings similarly as the group in the previous part of the study. The schedule of the blood drawings was: 12:00, 12:30, 13:00 and 13:30.

Blood was taken into a Vacuette Serum Clot Activator tubes (a plastic tube with a clotting activator and separation gel). Serum was obtained by centrifugation for 5 minutes at 2000 *g* at 4 °C, and stored at –20 °C.

Cortisol, cortisone, corticosterone and aldosterone were measured by LC-MS/MS (Sosvorova et al., 2015), and cortisol was additionally measured using an RIA kit from Immunotech (Czech Republic).

Statistical analyses

The relationships between dependent variables and the effects of sampling time were evaluated using a repeated measures ANOVA model consisting of the following factors: Time (10:00, 10:15, 10:30, 10:45 and 11:00 for experiment 1); (11:00, 12:00, 12:30, 13:00 and 13:30 for experiment 2) and Subject (explaining inter-individual variability). The ANOVA model was followed by least significant difference (LSD) multiple comparisons. To eliminate skewed data distribution and heteroscedasticity, the original data were transformed by Box-Cox transformation to attain a Gaussian distribution and constant variance before further processing. Simple regression was used to compare the two different analytical methods, with the RIA method chosen as the reference since it is commonly used during routine cortisol measurements. The statistical software Statgraphics Centurion, version XV from Statpoint Inc. (Herndon, Virginia, USA) was used for all statistical analyses.

Results

Stress

Figure 1 shows the profile of cortisol levels when cannulation was being performed as well as when cannulation had been performed 150 minutes before the first blood sampling. Values just after inserting the cannula were significantly higher than values when calm. This reflects the fact that blood drawing can invoke minor or even fairly high stress in some patients. Higher levels of plasma cortisol lasted at least 1 hour after the first sampling. These results indicate that merely the knowledge that blood will be drawn can stimulate higher cortisol levels, not just the needle insertion itself. Cortisone levels were also increased, similarly as for cortisol (Figure 1).

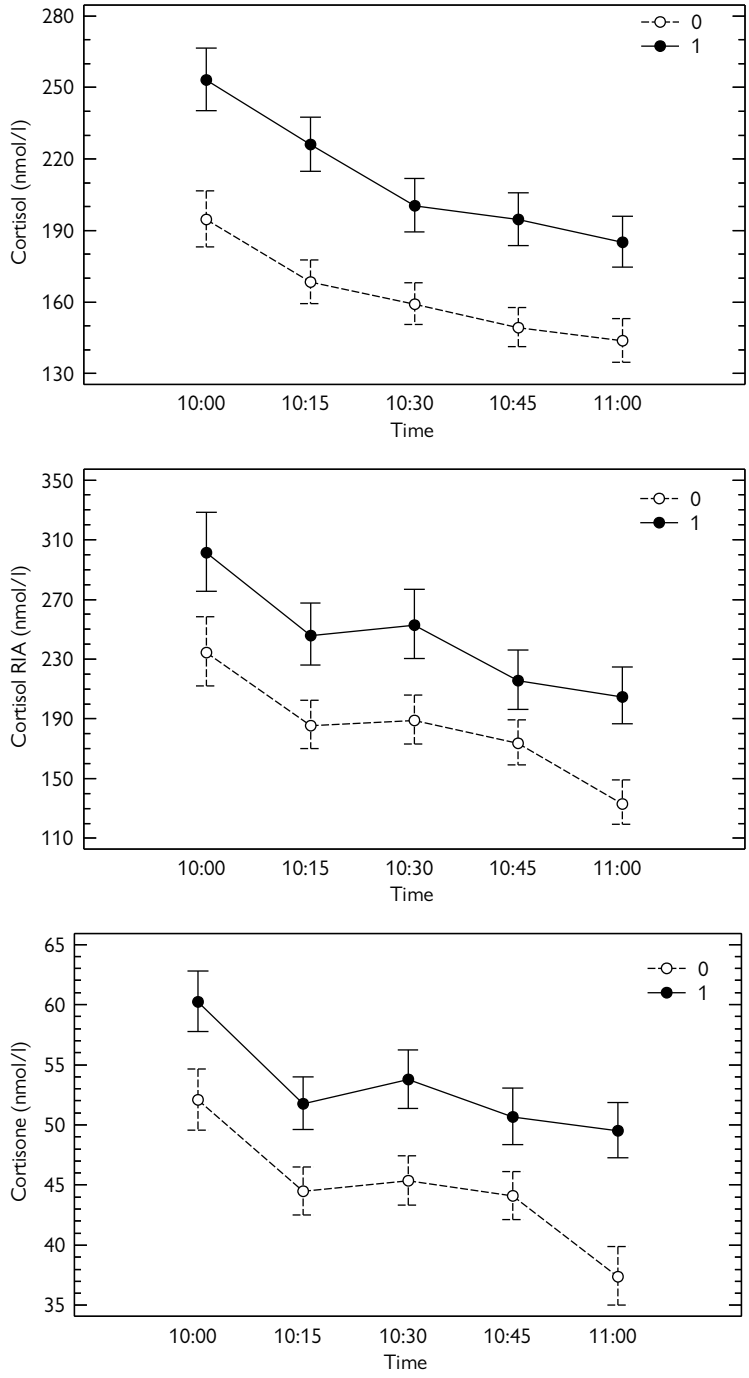

Figure 1 – Influence of the time of blood drawing after cannulisation on cortisol levels (measured LC-MS/MS and RIA) and cortisone (0 – cannula inserted at 8:00; 1 – cannula inserted at 10:00).

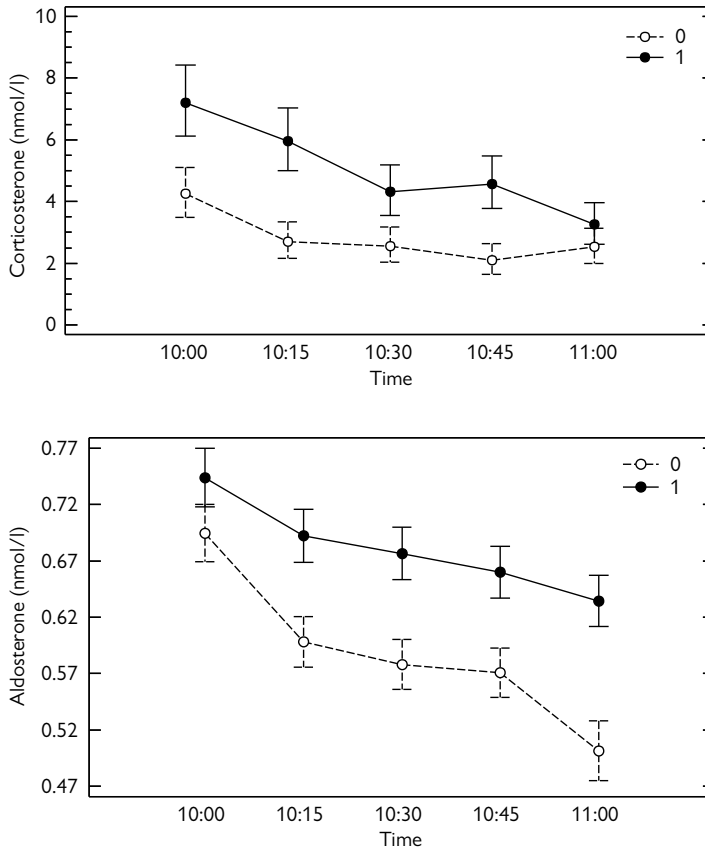

Figure 2 – Influence of the time of blood drawing after cannulisation on levels of corticosterone and aldosterone (0 – cannula inserted at 8:00; 1 – cannula inserted at 10:00).

Corticosterone was also increased already at the time of sampling, while increases in its metabolite aldosterone came later (Figure 2). As opposed to the other corticoids tested, the effect of stress on corticosterone disappeared after an hour (Figures 1 and 2).

The main lunchtime meal

The effects of the main meal of the day – lunch at noontime – were interesting. All corticoids tested had a marked increase between 11 and 12 o'clock, which could reflect a physiological preparation for eating as part of the circadian rhythm. After eating there was an evident decline in cortisone levels, while its precursor cortisol had rather a plateau in its decline (Figure 3). Similarly, there was an evident decline in aldosterone but a plateau in the decline of its precursor corticosterone (Figure 4).

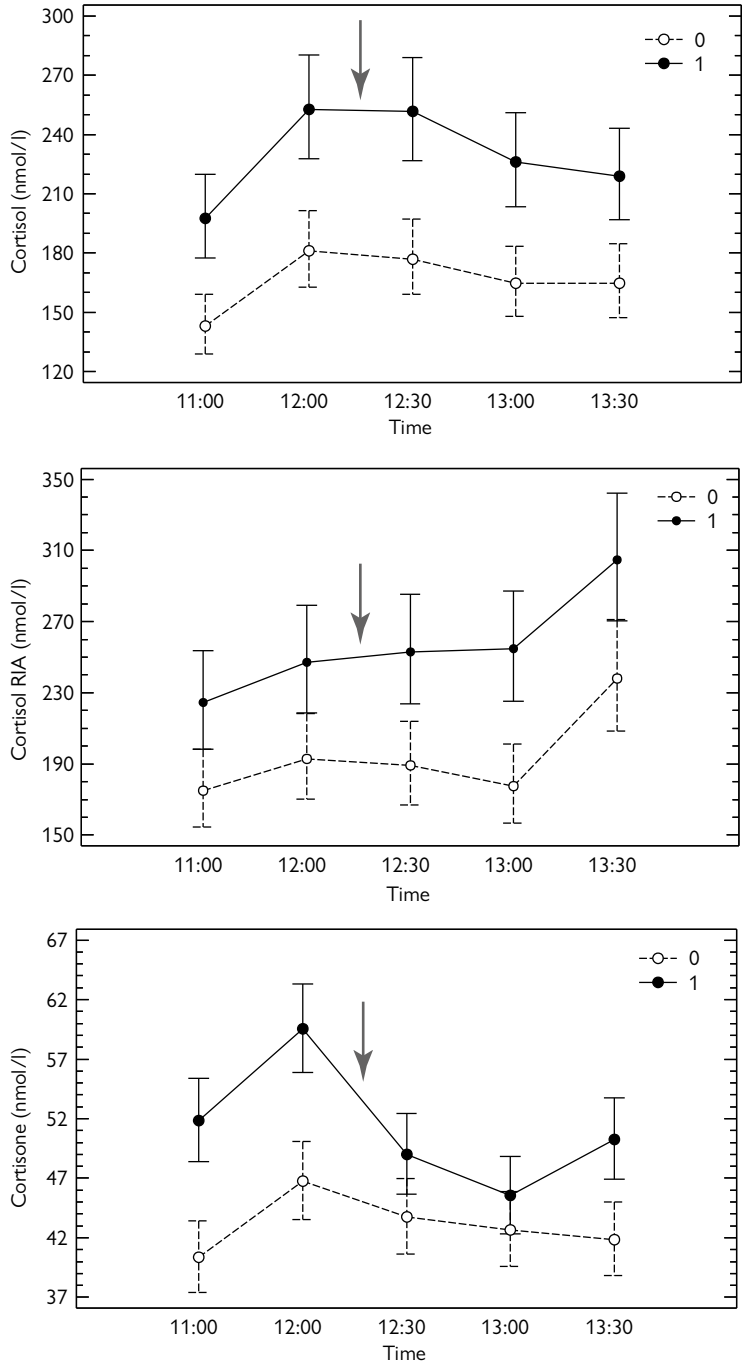

Figure 3 – Cortisol (measured LC-MS/MS and RIA) and cortisone levels before lunch and then at half-hour intervals (0 – with no lunch; 1 – lunch eaten after sampling at 12:00; ↓ – lunch).

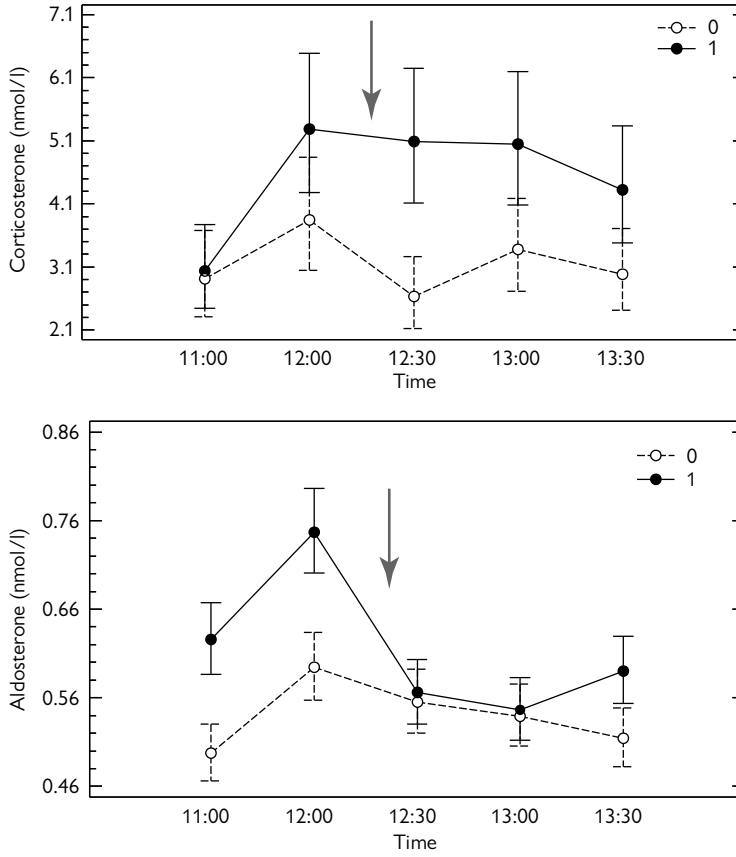

Figure 4 – Levels of corticosterone and aldosterone before lunch and then at half-hour intervals after lunchtime (0 – with no lunch; 1 – lunch eaten after sampling at 12:00; ↓ – lunch).

Choice of analytical method

We compared cortisol in 90 plasma samples measured by the commercial RIA kit from Immunotech and a published LC-MS/MS method (Sosvorova et al., 2015). The RIA method, which was used as the reference method, showed strong correlation with the LC method (r=0.85), with the regression approximated by the equation y = 0.650x + 49.62 (Figure 5). The slope of the regression line indicates some overestimation of cortisol levels when using RIA.

Discussion

In our study we focused on cortisol and its main metabolite cortisone as the primary stress hormones, and on aldosterone as the main mineralocorticoid. We also analysed corticosterone, even though it is a minor glucocorticoid in humans, as opposed to rodents and other vertebrates; it has been hypothesised, however, that

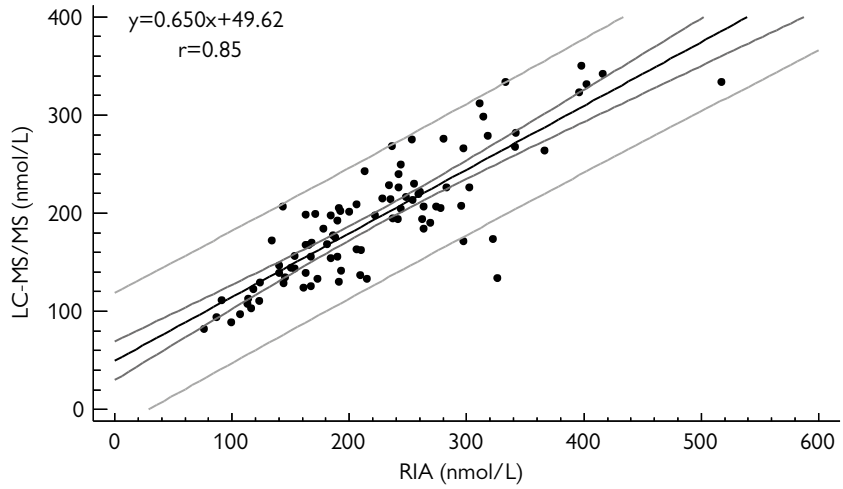

Figure 5 – Comparison of the LC-MS/MS and RIA methods. Simple regression for cortisol. RIA was selected as the reference method and LC-MS/MS as the test method.

under certain physiological conditions corticosterone has additional biochemical functions over and above those of cortisol (Morris, 2015).

Stress is a common reaction to having blood drawn, brought on either directly by the insertion of the needle or even more so by the fear that precedes it. Such emotional excitement can leave traces on physiological functions even two hours after the stimulus abates (Pieper et al., 2007). Here we demonstrate that a stress reaction can precede cannulation and be evident in samples taken during the cannulation procedure. Further increases in cortisol after cannulation were not found, but the subsequent decline was slow and higher levels were found even an hour later.

For a correct interpretation of results, therefore, a calm environment during blood drawing and a sufficiently calm patient are necessary. For clinical studies or research purposes, we recommend taking samples from a cannula that has been inserted 2 hours before the actual blood sampling.

Using salivary cortisol could be completely eliminated the problem of the stress caused by blood sampling. Salivary cortisol represents an easy method and permits possible samplings with high frequency, during the normal activity of patients at home. This method is promising for the future, but needs more data of physiological and normal values (Kosák et al., 2014).

There is as yet little information about the influence of food intake on the daily profile of cortisol. Using 15-minute intervals, Knoll et al. (1984) found higher cortisol levels after lunch compared to after dinner. Goldman et al. (1985) found peak cortisol during both the lunch and dinner periods. The timing of food

intake is also important (Follenius et al., 1982; Simonetta et al., 1991; Van Cauter et al., 1992; Elimam and Marcus, 2002; Bandin et al., 2015). Studies mostly agree that higher levels occur after food intake, but increases do not occur constantly. Higher cortisol levels after food have even been found in saliva (Toda et al., 2004).

In our study we found physiologically slightly increased cortisol before 12 o'clock, which is the traditional time in the Czech Republic for the main meal (lunch). This could be a physiological reaction to preparing for food intake. This increase was also evident for the other corticoids (cortisone, aldosterone, corticosterone). However, individual corticoids reacted differently to the lunch, with plateaus in the levels of cortisol and corticosterone but continuous declines in the levels of their metabolites (cortisone, respectively aldosterone).

The choice of analytical method

For standard clinical biochemistry, immunoassays are certainly sufficient. It must be kept in mind, however, that in some cases values can be significantly distorted. One example is in women who are taking some types of hormonal contraceptives (Šimůnková et al., 2008). This distortion is partly a result of cross-reactions of antisera for cortisol with products of the metabolism of some gestagens present in combined contraceptive formulas.

For scientific studies and partly for clinical studies it would be more correct to measure cortisol after chromatographic separation, preferably liquid chromatography with mass spectrometry (LC-MS). Values obtained by LC-MS better reflect actual concentrations of circulating cortisol and are statistically significantly lower than values from radioimmunoassay. Another advantage of LC-MS is the possibility to measure multiple steroids in one assay, enabling a more complex perspective. On the other hand, the fact that immunoassays are more accessible for smaller laboratories where mass spectrometry would be non-economical is an argument for the use of RIA. Therefore, with proper standardisation and awareness of the method's limitations, RIA remains the first choice for routine analyses (Taylor et al., 2015).

Conclusion

Not just endocrinologists but also other specialists often require cortisol measurements from biochemical laboratories. Determining the correct levels of cortisol, especially in the differential diagnosis of hypocortisolism, hypercortisolism and normal functioning of the hypothalamic-pituitary-adrenal axis, require maintaining the proper conditions even in the pre-analytical phase of sampling. It is necessary to take into account the daily rhythm of cortisol and avoid sampling in the first hours after waking (if not specifically measuring CAR), as well as taking into account food intake and the stress of blood drawing. Finally, the choice of proper analytical method should be made with knowledge of their limitations.

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The Cortisol to Cortisone Ratio during Cardiac Catheterisation in Sows

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Abstract: A possible effect of mini-invasive heart intervention on a response of hypothalamo-pituitary-adrenal stress axis and conversion of cortisone to cortisol were studied. We have analysed two stress markers levels (cortisol, cortisone) and cortisol/cortisone ratio in 25 sows using minimally invasive heart catheterisation as the stress factor. The values of studied parameters were assessed in four periods of the experiment: (1) the baseline level on the day before intervention, (2) after the introduction of anaesthesia, (3) after conducting tissue stimulation or ablation, and (4) after the end of the catheterisation. For statistical analyses we used the non-parametric Friedman test for four dependent samples (including all four stages of the operation) or three dependent samples (influence of operation only, baseline level was excluded). Statistically significant differences in both Friedman tests were found for cortisol and for cortisone. We have found the highest level of cortisol/cortisone ratio in unstressed conditions, then it decreased to the minimal level at

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the end of the intervention. We have concluded that cortisol levels are blunted by the influence of anaesthesia after its administration, and therefore decrease back to the baseline at the end of the operation.

Introduction

Stress and stress marker detection has been extensively discussed for many years in both human and veterinary medicine. Stimuli affecting the homeostasis of organism are called stressors and arise from many different origins e.g. ecological (acute environmental changes, absence of nutrition or shelter, temperature variations), sociobiological (unstable social hierarchy), health (infection, injury, surgery), transport and many others. Stressors trigger a stress response – a complex of physiological, endocrinal, metabolic and behavioural reactions protecting the organism from the injurious effect (Schreiber, 1985; Greenberg et al., 2002; Möstl and Palme, 2002). In our presented study we have studied the stress response of young sows to an invasive heart catheterisation which was supposed to be a stressor. In order to evaluate the stress response of hypothalamo-pituitary-adrenal axis concentration of the adrenal cortex steroid (cortisol, cortisone) was determined as well as the cortisol/cortisone ratio.

Cortisol's molecular structure is lipophilic, allowing the unbound cortisol to enter freely the target cells through the cell membrane into the cytoplasm where it is bound to specific receptors. The cortisol-receptor complex then enters the nucleus and identifies glucocorticoid response elements (GREs), special palindromic DNA sequences, binds to them, and then acts as a transcription modulator (Seckl, 1997).

Cortisone is a steroid hormone also produced by the adrenal cortex. This hormone is characterised by its possession of a keto-group on C-11 (cortisol has hydroxyl group on C-11) and cannot be bound to cytoplasmatic receptors. Cortisone functions as a reserve pool of cortisol, providing more cortisol when needed (e.g. in stress response). Two isoenzymes of 11β-hydroxysteroid dehydrogenase (11β-HSD) act as important regulation factors for the conversion of cortisone to cortisol (11β-HSD1) and conversely cortisol to cortisone (11β-HSD2). 11β-HSD1 amplifies glucocorticoid action in the liver, adipose tissue, inflammatory cells, and vasculature, providing a therapeutic target for inhibition in type 2 diabetes. 11β-HSD2 limits cortisol action, and thereby facilitates aldosterone action in the distal nephron and a few other sites (Kilgour et al., 2015).

The aim of the presented study was to determine stress marker levels including cortisol/cortisone ratio in each period of the experiment – we compared the level obtained at the breeding farm (under non-stress conditions) with levels obtained during the heart catheterisation experiment (potential stress events). Our second aim was to determine, if there are any differences in stress markers levels during minimally invasive heart intervention itself (the first blood collection was excluded).

We hope our findings could help improve elective cardiac procedures to minimise their effect on human patients or animal recipients.

Material and Methods

For this study, serum concentrations of two adrenal activity markers (cortisol, cortisone) were determined in sows undergoing elective heart catheterisation. Although it was only minimally invasive surgery, this provided a stress overload event. Marker levels were measured during four well defined periods of the experiment (details were published elsewhere – Skarlandtová et al., 2012, and they are shortly described in section "Blood collection") in order to evaluate any changes in their concentrations. Our aim was to determine if elective minimally invasive intervention has an influence on the hypothalamo-pituitary-adrenal (HPA) axis and consequently on the activity of the adrenal cortex.

The experiment was performed in accordance with Czech law and corresponding EU regulations and was approved by the Institutional Animal Care and Use Committee.

Animals

Twenty-five four month old sows (*Sus scrofa domestica*) were used in the experiment, from the crossbreed Landrace × Large White, details of breeding, housing, etc. of animals were described in our previous publication (Skarlandtová et al., 2012). The sows were in prepubertal age; therefore we can exclude the estrous cycle influence on the stress marker levels.

Experiment

The effect of heart catheterisation on stress marker levels in blood serum was tested. Changes in blood serum concentrations of cortisol and cortisone were determined.

Heart catheterisation

Heart catheterisation was performed following the standard catheterization procedure (through arteria and vena femoralis using a 7-9F sheet). The catheterizations were carried out within the frame of electrophysiological projects, where cardiac stimulation or conducting tissue electrical radiofrequency ablation was performed. In all tested animals two markers were assessed in the blood serum: cortisol and cortisone.

Anaesthesia and medication

Stresnil (5 mg/kg), atropin (0.05 mg/kg) and narcetan (14 mg/kg) were used via intramuscular injection for pre-medication and sedation. An 18G or 20G IV cannula was inserted into the marginal ear vein to obtain intravenous (IV) access. Intravenous anaesthetic introduction was initiated using a propofol (2 mg/kg) and

morphine (0.2 mg/kg) bolus. Intubation under direct laryngoscopic control was performed with 7 or 7.5 mm orotracheal tubes, depending on the size of the sow. Anaesthesia was maintained with a propofol (4 mg/kg/h) IV infusion, and as an analgesic, a morphine (0.2 mg/kg) IV bolus was administered every hour. Ventilation was sustained at an average volume of 8 to 10 ml/kg and respiratory rates 15 per minute. No inhalation anaesthesia was used.

During the IV anaesthesia a continuous monitoring of mean arterial pressure (MAP), heartbeat rate (HR), O_2 saturation (SO_2) and exhalated capnometry (PCO_2) was observed on a multiparameter bed-side monitor.

Blood collection

Blood was collected from the jugular vein during each of the four defined periods of the experiment. The first (1) was collected at the farm, in non-stress domestic conditions (control sample, baseline stress marker levels), other samples were collected 10 minutes after the presumed stress situation: a second sample (2) 10 minutes after intubation and the introduction to anaesthesia, a third sample (3) 10 minutes after cardio stimulation or conducting tissue ablation and the last (4) at the end of the intervention, before the animal was sacrificed. Blood samples (10 ml) were collected in 10 ml serum Vacutainer system tubes (BD Vacutainer, SSt II Advance), and after a 30 minute incubation at room temperature were centrifuged (2000× *g*) for 15 minutes and then serum was stored at –20 °C until later analysis. The whole experiment can be divided into two sections: the first period (non-stress conditions, baseline reference sample) and the second, surgery section (the second, third and fourth periods of the experiment) (Figure 1).

Laboratory analyses

Cortisol and cortisone were measured using the method published elsewhere (Šimůnková et al., 2008). In brief, the serum samples were twice extracted and then the hormones separated using a high performance liquid chromatography (HPLC)

Figure 1 – *The blood collection system. Arrows indicate the blood sampling in four defined parts of the experiment. For the first time, sows were blood sampled at the breeding farm, in non-stress conditions, after twenty-four hours were sows transported to the experimental laboratory, and then were blood sampled after intubation and introduction to anaesthesia (second sample), after cardiostimulation or conducting tissue ablation and the last, at the end of the catheterisation.*

system from Dionex Softron (Germering, Germany). HPLC separation was carried out with reverse phase EC 250/4 NUCLEOSIL® 100-5 C18 column (MACHEREY-NAGEL, Düren, Germany), and to avoid possible column contamination the Phenomenex SecurityGuard system with cartridge C18 (Phenomenex, Torrance, CA) was used. Merck (Darmstadt, Germany) solvents were used as the mobile phase for HPLC. Cortisol and cortisone concentrations in the serum were determined according to a calibration curve using UV/VIS detection.

Statistics

As our data did not have a standard Gaussian distribution, non-parametric statistical methods were used to analyse the differences in stress marker levels within the experiment. For testing difference in stress markers levels within the experiment non-parametric Friedman test was used. Friedman test was also calculated for determining cortisol/cortisone ratio for four and three dependent samples.

Results

Elementary statistical data was calculated for the measured markers (cortisol, cortisone) for each period of the experiment (1–4). A wide variance in measured data was demonstrated, indicating substantial inter-individual differences in the assessed marker levels; for illustration, box and whisker plots (Figures 2–4) are shown for each marker. Medians (for exclusion outlier values) were noted and discussed, and the results shown separately for each marker.

Cortisol

As we expected, the serum cortisol concentration was the lowest (148.35 nmol/l) in the non-stress sample at the farm. Therefore we can regard the samples from the first period of the experiment as the baseline level. The cortisol concentration

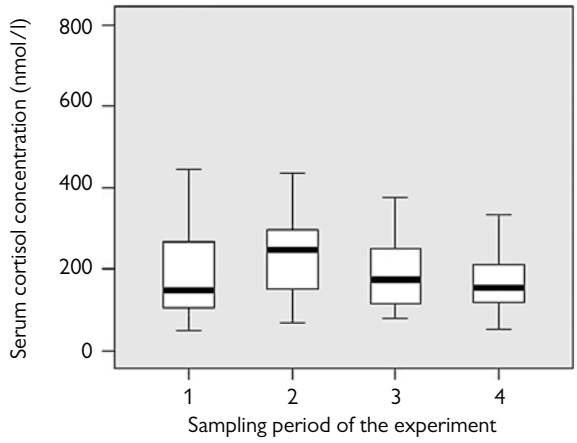

Figure 2 – Box and whisker plot: Serum cortisol concentrations during the four sampling periods of the experiment. There are 50% of measured values of cortisol concentration in the box, the median is marked as a bold line in the box, whiskers are 25% measured values.

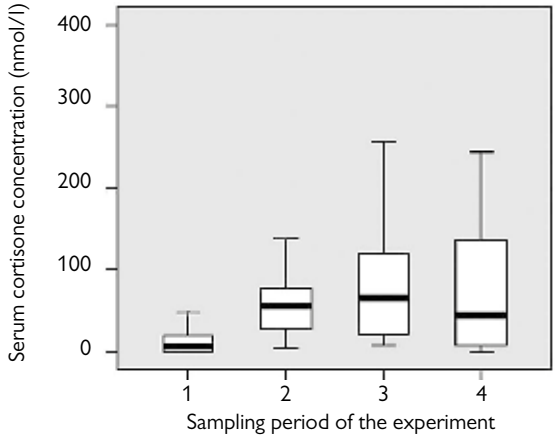

Figure 3 – Box and whisker plot: Serum cortisone concentrations during the four sampling periods of the experiment. There are 50% of measured values of cortisone concentration in the box, the median is marked as a bold line in the box, whiskers are 25% measured values.

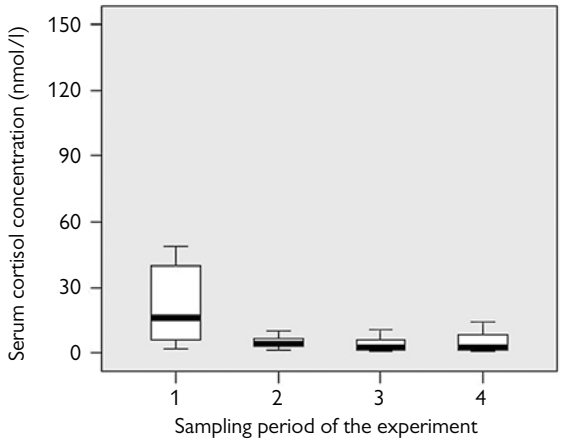

Figure 4 – Box and whisker plot: Serum cortisol to cortisone ratio during the four sampling periods of the experiment. There are 50% of measured values of cortisone concentration in the box, the median is marked as a bold line in the box, whiskers are 25% measured values.

then increased to the highest level (246.41 nmol/l) during the second period of the experiment (after the introduction anaesthesia). During the third and fourth periods of the experiment, cortisol levels decreased to concentrations close to the baseline (175.42 nmol/l and 154.30 nmol/l respectively).

The statistics (Friedman test) were significant ($p<0.05$) in both tests – for all four sampled periods of the experiment, and for the three stages excluding the first one. This suggests that the surgery section itself also had an important influence on the levels of cortisol.

Cortisone

Similar to the cortisol median levels, the concentration of cortisone was the lowest during the first period of the experiment (6.19 nmol/l). Levels increased during the second and third periods (the highest level was recorded during the

third sampling time; 66.12 nmol/l). The cortisone level then decreased at the end of the intervention, but was still seven times higher than the baseline (44.70 nmol/l). Statistics for all periods of the experiment were significant ($p<0.001$), and, if we exclude the first stage of the experiment, statistics were $p<0.05$. This means that the cortisone levels statistically changed in all periods of the experiment, and also for the three stages of heart catheterisation when viewed separately from the non-stress (baseline) period.

Cortisol/cortisone ratio

Cortisol/cortisone ratio was calculated for each pig separately, median, minimum and maximum for each period of the experiment was determined. Median was the highest for the first period of the experiment (non-stress conditions) (15.95), then rapidly decreased and the lowest value was determined at the end of the surgery (2.51) (Table 1).

Friedman test for this ratio was statistically significant only for surgery periods of experiment compared to the baseline level under unstressed conditions ($p<0.001$).

Discussion

Our study was focused on the HPA (hypothalamo-pituitary-adrenal) axis activity, but not on that of the SAM (sympatho-adreno-medullar) axis. The first and the most important reason was the fact that catecholamines (unlike glucocorticoids) are released in a few seconds following the stress stimulus, and the design of our experiment technically disallowed the blood collection in this very short time. Moreover it is unable to assess catecholamines and glucocorticoids (and other tested markers) in the same blood sample and two blood collections at every stage of the experiment could cause two problems: 1) the volume of the collected blood and 2) possible affection of the second sample results by the preceding blood collection.

Anaesthesia influence on adrenal hormones secretion

Anaesthesia is used to minimize the traumatic effect of surgical procedures. It is well known that anaesthesia blunts stress response through the suppression of

Table 1 – Ratio of cortisol/cortisol concentrations

Sampling period of experiment[1]	Median	Minimum	Maximum	p
1	15.95	1.71	144.75	<0.001
2	4.48	1.17	32.65	
3	2.61	0.44	17.37	
4	2.51	0.73	20.38	

[1]sampling period of the experiment: 1) the baseline level at non-stress conditions at home farm; 2) after intubation and introduction to anaesthesia; 3) after cardiac stimulation or conducting tissue ablation; 4) at the end of experiment

the secretion of stress hormones (catecholamines and glucocorticoids). There are divergent types of anaesthesia with different suppression rates of stress response – balanced (inhalation) and total intravenous anaesthesia (TIVA). Many studies have found lower stress marker levels in TIVA (a combination of propofol and an opioid as an analgesic component) compared to balanced anaesthesia (a combination of inhalation gases e.g. sevoflurane, isoflurane, enflurane, etc. and an opioid) (Schricker et al., 2000; Ledowski et al., 2005; Ihn et al., 2009; Kostopanagiotou et al., 2010; Marana et al., 2010). TIVA, consisting of a combination of propofol and morphine, was used in our experiment (for details see in section "Anaesthesia and medication"). Propofol is lipophilic weak acid with voltage-gated ion L-calcium channels in heart influence. It improves decreased sympathetic activity alpha and beta adrenergic receptors (Krzych et al., 2009). Combination of propofol and morphine (or other opioid) decreasing catecholamines and glucocorticoid released to blood (Fragen et al., 1987; Schricker et al., 1999, 2000; Ihn et al., 2009).

The propofol/opioid combination suppresses HPA activity at each level. That is, the suppression of the production of the corticotrophin releasing hormone (CRH), adrenocorticotropin hormone (ACTH) and the glucocorticoids. This could be caused by a synergic propofol-opioid reaction on the hypothalamic receptors that suppress noxious afferent stimuli and then suppress CRH release (due to the increase in the GABA receptor inhibitor concentration) (Kostopanagiotou et al., 2010; Marana et al., 2010).

Many authors (e.g. Van Hemelrijck et al., 1995; Han et al., 2012; Offinger et al., 2012) found decreased cortisol levels 45 minutes after the introduction of anaesthesia, which corresponds with our results. Stress steroid levels were lower in comparison to non-stress ones (e.g. Fragen et al., 1987; Schricker et al., 1999, 2000; Ihn et al., 2009). In our experiment, the results for cortisol concentration corresponded to referenced authors, with the highest levels determined in the second period of the experiment i.e. a very short time after the introduction to anaesthesia, then during the third and fourth periods the levels decreased approximately back to the baseline concentration. In comparison, cortisone concentrations increased during the second period of the experiment and were higher compared with the baseline levels. We attribute this to the balance stress reaction of these steroids.

Surgical procedure

In this experiment the minimally invasive surgical procedure of heart catheterization was used. A number of previous studies found no difference in elevated cortisol levels between invasive open surgery and minimally invasive procedures in pigs (Mansour et al., 1992; Bessler et al., 1994; Burpee et al., 2002; Margulis et al., 2005; Matsumoto et al., 2005; Duchene et al., 2008). These findings could mean that even minimal intervention can cause a rise in cortisol

levels. However, stress response to surgery could be modulated by some other parameters, e.g. type of anaesthesia (mentioned above), handling, etc. Contrastingly, experimental measurements in human patients have found some differences in stress response for the same type of the laparoscopic intervention using a slightly different surgical approach (Han et al., 2012).

Our findings suggest that the most stressful stage of the heart catheterization is during its very beginning. This fact should be considered in invasive animal experiments but it would be very difficult to transfer this experience to human cardiac interventions particularly because of standard sedative premedication of human patients.

Cortisol and cortisone

Basic levels of cortisol in our group of experimental animals differ from some data reported previously (e.g. Perremans et al., 2001). The difference can be explained by many factors: different environmental conditions of live, procedure of cannulation, different genetic groups of animals, and big interindividual differences among individual animals. This was one of reasons why we had to measure our own basic levels of examined hormones and did not rely on literature data.

Serum cortisone concentration in our experiment was at the lowest level during the first period of the experiment, then it increased to the highest levels during the third period, and during the fourth period it slightly decreased. This corresponds with the cortisol level, which was found to be at the highest concentration after exposure to anaesthesia and then it decreased. We assume that the higher levels of cortisone were caused as a result of the higher activity of the 11β-HSD2 isoenzyme (the crucial regulation factor of cortisol/cortisone levels), in this way balancing the action of the cortisol.

Cortisone and cortisol/cortisone ratio

Cortisone could be considered to be an inactive form of cortisol, but, on the other hand, it represents cortisol's reserve pool in the time when more cortisol is needed (e.g. in the stress response). The serum cortisol/cortisone ratio isoenzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) balanced. The activity of these isoformes is crucial in cortisol/cortisone levels in blood serum. The mainly mechanisms in this role of these isoenzymes is presumably cortisol inactivation by 11β-HSD2 in kidneys (higher activity caused cortisol inactivation) and 11β-HSD1 in the liver (cortisone restoration) (Vogeser et al., 2003).

Conclusion

In our experiment cortisol, cortisone and cortisol/cortisone levels were determined during four stages of the heart catheterisation of young sows. We separately calculated Friedman tests for each marker during the four defined periods of the experiment; these tests were statistically significant for all markers.

The lowest concentration levels for both markers were measured during the baseline, non-stress conditions. Therefore, we can conclude that the conditions at sows' home farm are non-stressful. From the baseline, the serum concentrations of both markers then increased to their highest levels; for cortisol during the second period and for cortisone during the third one. We can assume that the adrenal secretion of cortisol occurs in response to the most stressful conditions before the exposure to intravenous anaesthesia, and then the anaesthesia minimizes the stress response. The other marker, cortisone, acts as a balanced system against the traumatic effects of stress. We have found the highest level of cortisol/cortisone ratio in unstressed conditions, then it decreased to minimally level at the end of the intervention, it could be due to anaesthesia minimizing the stress response and cortisol levels decreased after exposure the anaesthesia and cortisone levels were higher against baseline.

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How Does Energy Intake Influence the Levels of Certain Steroids?

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Abstract: The influence of steroid hormones on food intake is well described. However, there are only a few studies on the effect of food intake on steroid levels. The study involved eight non-smoker women (average age 29.48 ± 2.99 years; average BMI 21.3 ± 1.3 kg/m^2); they did not use any kind of medication affecting steroidogenesis. We analysed the influence of four various stimuli on the levels of steroid hormones and melatonin. During their follicular phase of menstrual cycle, each woman had an oral glucose tolerance test (OGTT), intravenous glucose tolerance test (IVGTT), a standard breakfast and psyllium (a non-caloric fibre). Cortisol declined during each test, which is a physiological decline in the morning hours. In all tests (except of the application of the non-caloric fibre, psyllium), however, this decline was modified. After the standard breakfast there was an increase in cortisol at 40th minute. The OGTT and IVGTT tests led to a plateau in cortisol levels. Testosterone levels and those of other steroid hormones showed no relationships to tested stimulations. Oral and intravenous glucose have influenced physiological decline of melatonin levels. During the IVGTT test, melatonin levels started to increase at 20th minute, reaching a maximum at 40th minute. The OGTT test led to a delayed increase in melatonin levels, compared to IVGTT. Despite the fact that we performed the tests in the morning hours, when steroid hormone levels physiologically start to change due to their diurnal rhythm, we still found that food intake influences some of the hormone levels.

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Introduction

Steroid hormones are hormones with lipophilic character synthesized from cholesterol in the gonads and adrenal glands, as well as locally in other tissues such as the brain (neurosteroids) and the placenta. In the plasma they are mainly bound to plasma proteins (albumin, or specific globulins such as corticosteroid-binding-globulin (CBG) or sex-hormone-binding globulin (SHBG)), and only a minor proportion (1–10%) of hormones are found in free form. In target tissues they can act through two mechanisms: genomically through intracellular receptors, with function taking on the order of hours to days but also having lasting influence; and non-genomically, with rapid onset of function on the order of minutes or even seconds. This latter mechanism is typical for the activity of neuroactive steroids on nerve synapses, or the interaction of steroids with the GABA-A receptor.

Even though the daily profiles of the main sex hormones are well known, small oscillations in their levels that may be connected to food intake have yet to be systematically studied. Glucocorticoids have many various functions in organisms, and they influence the maintenance of the homeostasis. With rhythmic changes of the hormone levels, the hypothalamic-pituitary-adrenal (HPA) axis ensures tissue and organ-specific reactions of the organism to endogenous and exogenous stimuli (Lightman et al., 2008).

According to a study by Stárka et al. (2015), levels of DHEA and of its conjugated form androstenediol and 7α-hydroxy – DHEA decline one to two hours after lunch and dinner. Another recent study has described significant changes of estradiol and SHBG after food intake (Rácz et al., 2015). The influence of steroid hormones on the food intake is well known. In contrast, however, there are only a few published reports on how the food intake influences levels of steroid hormones.

Melatonin is a hormone that is associated with controlling of the circadian rhythm. It is mainly produced in the pineal gland during the night. The gastrointestinal tract seems to be the main source of melatonin during the daylight hours and also it is the main source of extrapineal melatonin. During the day, the gastrointestinal tract produces 400 times more melatonin than the pineal gland. This indicates the importance of melatonin in the pathophysiology of digestion.

The aim of our study was to analyse the influence of food intake on steroid hormone and melatonin levels. As stimuli we selected a standard breakfast, oral and intravenous glucose, and psyllium (as a model of mechanical effects of the food on the gastrointestinal tract).

Methods

The study participated eight women with average age of 29.48 ± 2.99 years and BMI of 21.3 ± 1.3 kg/m^2. All of the women were pre-menopausal, they were non-smokers, healthy, and they were not using any medication or hormonal contraceptive. Blood samples were collected during the follicular phase (days 1–7 of the menstrual cycle). Five days before they had undergoing the tests, all

of the women followed a standard protocol that did not vary much significantly from their normal daily routine (8 hours of sleep, food intake according to a standardized menu). Before the tests they were informed about the study protocol and they signed an informed consent form. The study was approved by the ethical commission of the Institute of Endocrinology in Prague.

Each woman passed the four different tests during four consecutive menstrual cycles:

1) OGTT – an oral glucose tolerance test – 75 g of glucose (Glukopur brand) in 250 ml of unsweetened tee perorally.
2) IVGTT – an intravenous glucose tolerance test – a bolus of 0.33 g of glucose per kg of weight in 20% intravenous solution, administrated to a peripheral vein.
3) A standard breakfast – two slices of bread, 50 g of breast-meat chicken slices, 1 slice of fresh cheese (total caloric content of the breakfast was 515 kcal, total protein content: 20.58 g, total carbohydrates: 47.75 g, total fat: 24.9 g).
4) Psyllium – a non-caloric fibre, which was meant to simulate mechanical stimulation of the gastrointestinal tract through distension. The women drank 4 g of psyllium in 250 ml water.

An intravenous cannula was inserted into the cubital vein ten minutes before the first blood sampling. Sampling was performed for 120 minutes, with the following schedule:

- the first sampling was performed at 7:30 a.m. after overnight fasting
- subsequent samplings were performed at 20, 40, 60, 90, and 120 minutes.

Analytical methods

Each sample was collected into a plastic tube containing 100 µl of 5% EDTA. Plasma was obtained after centrifugation for 5 min at 2000 rpm at 4 °C, then separated and frozen within half an hour of being drawn from the subject, and stored at –20 °C until analysed.

C-peptide was measured in serum using ECLIA (electrochemiluminiscence immunoassay, Modular E 170 analyser, Roche). The measuring range of the kit (defined by the lower detection limit and the maximum of the master curve) was 0.003–13.3 nmol/l or 0.01–40.0 ng/ml for plasma. Intra- and inter-assay coefficients of variation were 1.5% and 2.3%, respectively.

Blood glucose was measured using the enzymatic reference method with hexokinase (Cobas Integra 400 plus analyser, Roche). The measuring range of the kit was 0.12–40 mmol/l (2.16–720 mg/dl). Intra-set and inter-set reproducibility were 1.7% and 2.6%, respectively. Cortisol was measured using an RIA kit (Immunotech, France). Melatonin was measured using an RIA kit (Labor Diagnostika Nord GmbH and Co. KG, Germany). Sensitivity for melatonin was 2 pg/ml, intra-assay and inter-assay coefficients of variation were 9.8–12.1% and 9.6–12.3%, respectively.

Steroid hormones measured by a GC/MS method

The levels of 37 unconjugated steroids and their polar conjugates were measured in cubital vein blood using a GC/MS method (Hill et al., 2010). In brief, free steroids were extracted from plasma by diethyl-ether; steroid conjugates were hydrolysed and extracted. The resulting residues were derivatized by methoxyamine hydrochloride and analysed by GC/MS as follows.

Steroids were purchased from Steraloids (Newport, RI, USA), Sylon B from Supelco (Bellefonte, PA, USA), methoxylamine hydrochloride from Sigma (St. Louis, MO, USA) and solvents from Merck (Darmstadt, Germany).

Instruments

Measurements of steroid levels were done on a GCMS-QP2010 Plus system by Shimadzu (Kyoto, Japan) consisting of a gas chromatograph equipped with automatic flow control, an AOC-20s autosampler, and a single quadrupole detector with an adjustable electron voltage of 10–195 V. A capillary column with a medium polarity RESTEK Rxi phase (diameter 0.25 mm, length 15 m, film thickness 0.1 μm) was used for analyses. Electron impact ionization with electron voltage fixed at 70 V and emission current set to 160 μA was used. The temperatures of the injection port, ion source and interface were maintained at 220 °C, 300 °C, and 310 °C, respectively. Analyses were carried out in the splitless mode with a constant linear velocity of the carrier gas (He), which was maintained at 60 cm/s. The septum purge flow was set at 3 ml/min. The samples were injected using the high pressure mode (200 kPa), which was maintained for 1 min. The detector voltage was set to 1.4 kV.

Statistical data analysis

The changes of steroid levels and melatonin were evaluated using a repeated measures ANOVA model consisting of a Subject factor, explaining differences between subjects, and a Stage factor. Due to the non-Gaussian data distribution and non-constant variance, the original data were transformed by a power transformation to attain a symmetric distribution of the data and residuals as well as homoscedasticity (Meloun et al., 2000). The homogeneity of the transformed data was checked by residual analysis as described elsewhere (Meloun et al., 2002, 2004). Then, the significance of the values was evaluated by least significant differences multiple comparisons.

Results

In order to better elucidate various findings on the influence of food intake on hormone levels, we studied the influence of several stimuli on the course of hormone levels. As stimuli we chose a standard breakfast, oral glucose, intravenous glucose and psyllium (chosen to follow mechanical effects of food on the gastrointestinal tract). The timeline of samplings was focused on monitoring and

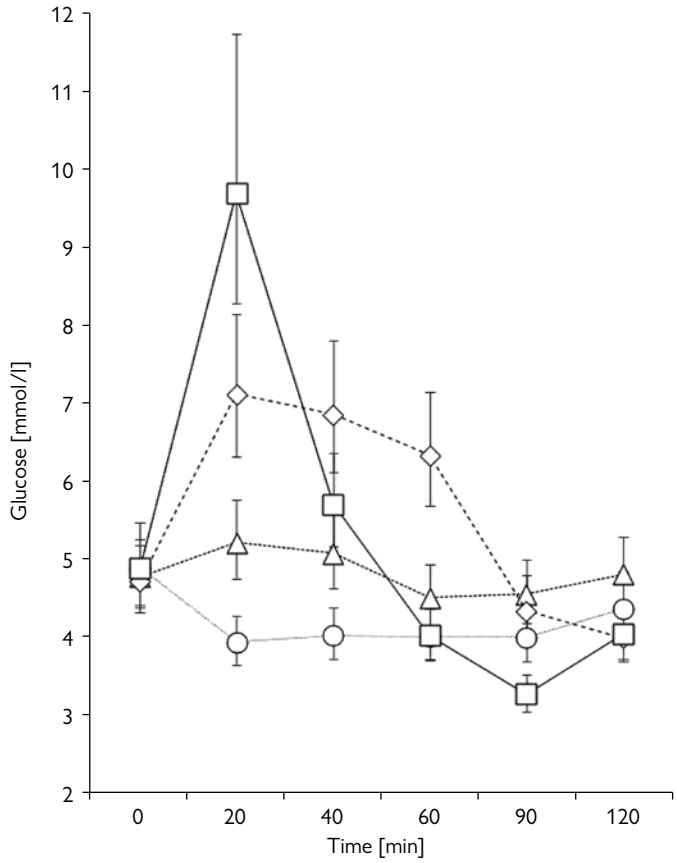

Figure 1 – Glycemia levels after individual stimuli.
Δ – standard breakfast; □ – IVGTT; ◊ – OGTT; ○ – psyllium

analysing of acute and small changes of hormone levels after each stimulus. As we expected, the glycemia and C-peptide levels reflected normal values of a healthy population (Figures 1 and 2).

Melatonin

After all stimuli there was a decrease in melatonin levels at the beginning of the test after the first sampling. During IVGTT there was an increase in melatonin levels 20 minutes after giving glucose, and this increase lasted up to 60 minutes. A similar increase occurred after oral glucose, though the increase was later than in case of intravenous glucose (Figure 3).

Cortisol

There was a slowing of the physiological decline in cortisol levels after each of the stimuli, excepting psyllium. This slowing was most pronounced after intravenous

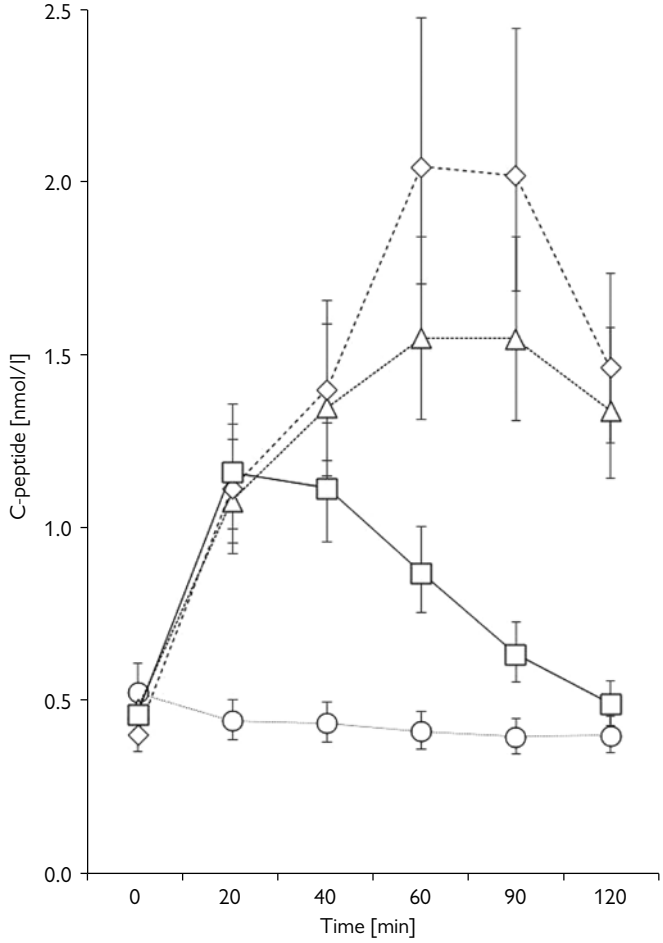

Figure 2 – C-peptide levels after individual stimuli.
Δ – standard breakfast; □ – IVGTT; ◊ – OGTT; ○ – psyllium

glucose, lasting even 60 minutes. After oral glucose and intravenous glucose there was a plateau in cortisol levels, but after breakfast there was an increase in cortisol at 40th minute (Figure 4).

DHEA

After the initial decline there was an increase in DHEA after all stimuli. This increase was most pronounced after intravenous glucose, but this increase was delayed compared to the other stimuli (Figure 5).

Testosterone

The course of testosterone levels did not have any significant relationship to any of the individual stimuli (Figure 6).

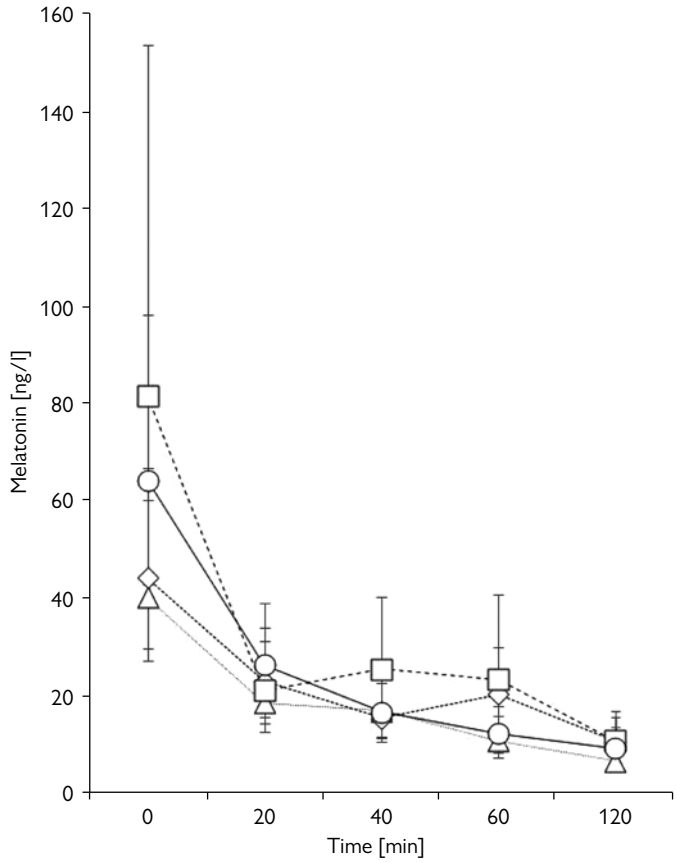

Figure 3 – Melatonin levels after individual stimuli.
Δ – standard breakfast; □ – IVGTT; ◊ – OGTT; ○ – psyllium

Similarly, non-conjugated and conjugated steroids also showed no relationships to individual stimuli, and we were unable to demonstrate a relationship between melatonin and the steroids studied.

Discussion

Recently, a number of studies have resulted in findings, about how dietary factors, such as caffeine (Sherman et al., 2011) and alcohol (Spanagel et al., 2005), can lead to changes of the circadian rhythm. It is assumed, that these changes are intermediated by melatonin (Peuhkuri et al., 2012). It is known, that starvation can lead to change of melatonin levels. In one study, restriction of energy intake for 2 days led to a decrease of plasmatic melatonin levels by 20%, while application of glucose during starvation led to a return of melatonin levels to the normal (Röjdmark and Wetterberg, 1989). In our study, we found a decrease in melatonin

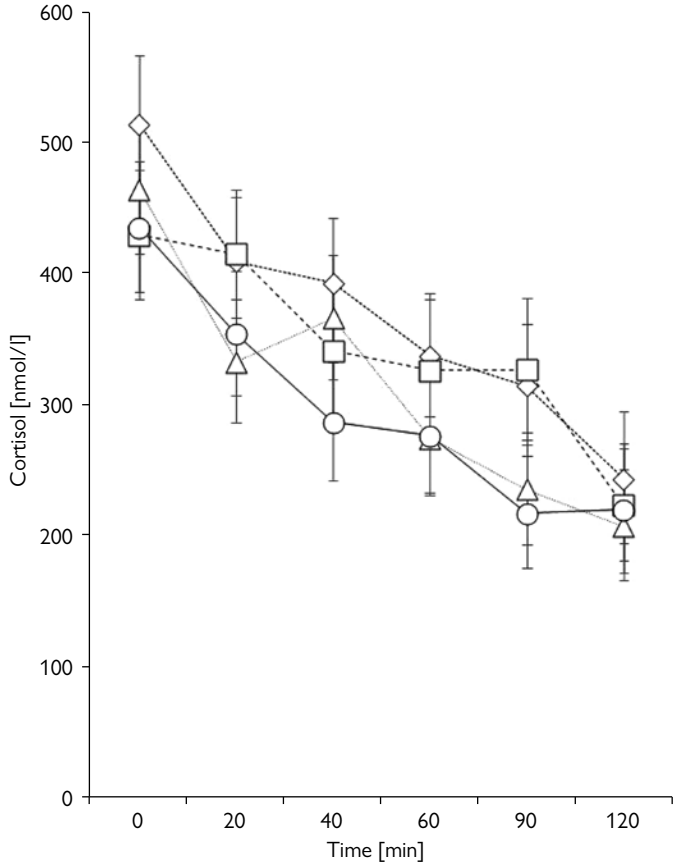

Figure 4 – Cortisol levels after individual stimuli.
Δ – standard breakfast; □ – IVGTT; ◊ – OGTT; ○ – psyllium

levels after the first blood samples were drawn, which reflects the physiological behaviour of the melatonin. Forty minutes after the intravenous application of the glucose there was an increase in melatonin, which lasted up to the 60th minute. Similarly, after giving of oral glucose there was an increase in melatonin levels, though delayed compared to its intravenous application. The reason is likely to the later onset of the hyperglycemia. In light of the fact, that neither the standard breakfast nor the non-caloric fibre led to a significant increase of the glycemia, it was either an increase of the melatonin levels observed.

It is well known the influence of corticoids on glucose metabolism and on increase of the glycemia (Lecocq et al., 1964). Some studies have described higher levels of circulating cortisol after each food intake (Follenius et al., 1982). According to a study from 1981 the most important stimulus for postprandial increased cortisol are proteins (Slag et al., 1981), while other studies showed similar effect of carbohydrates and lipids (Benedict et al., 2005; Stimson et al., 2014). A varied diet

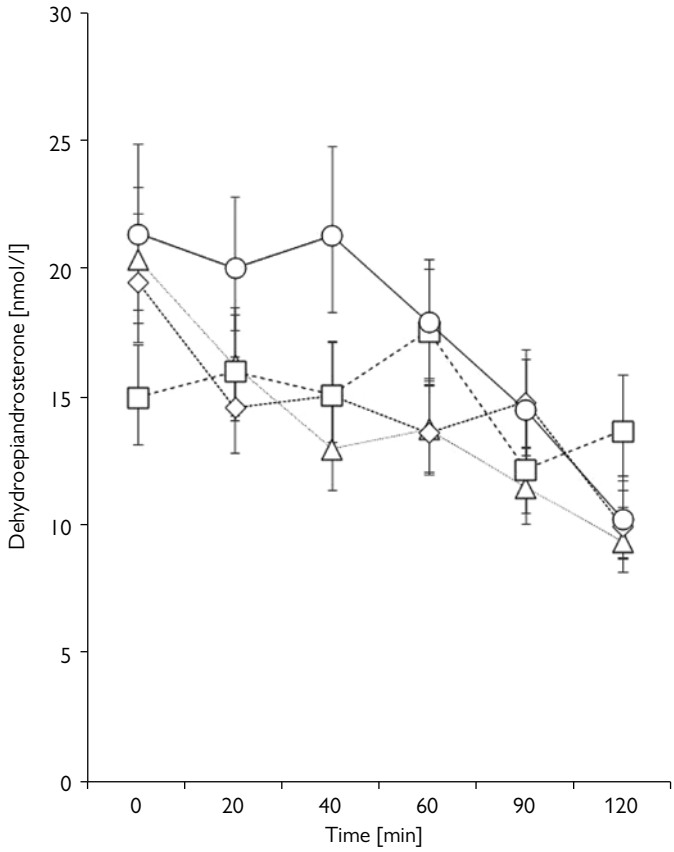

Figure 5 – DHEA levels after individual stimuli.
Δ – standard breakfast; □ – IVGTT; ◊ – OGTT; ○ – psyllium

and infusion of glucose and insulin (Wake et al., 2006) lead to changes of cortisone to active cortisol by activation of the enzyme 11β-hydroxysteroid-dehydrogenase 1 (Basu et al., 2004). According to Stimson et al. (2014), postprandial increase of cortisol is influenced not only by the increased production of cortisol from cortisone, but also by its increased secretion from the adrenal. In our study we found a delay in the physiological decline in cortisol levels connected to some of the stimuli. This delay was most pronounced after intravenous glucose admission and lasted until the 60th minute. After the breakfast there was an increase in cortisol levels at 40th minute, in accordance with the results of other studies.

The parenteral versus oral application of the studied stimuli can also play an important role. The postprandial increase of cortisol after oral nutritional stimulus is assumed to be influenced by signalling from the gastrointestinal tract (likely incretins, glucagon-like peptide 1, and gastric inhibitory polypeptide, which stimulate the hypothalamic-pituitary-adrenal axis (Herrmann et al., 1995). It is

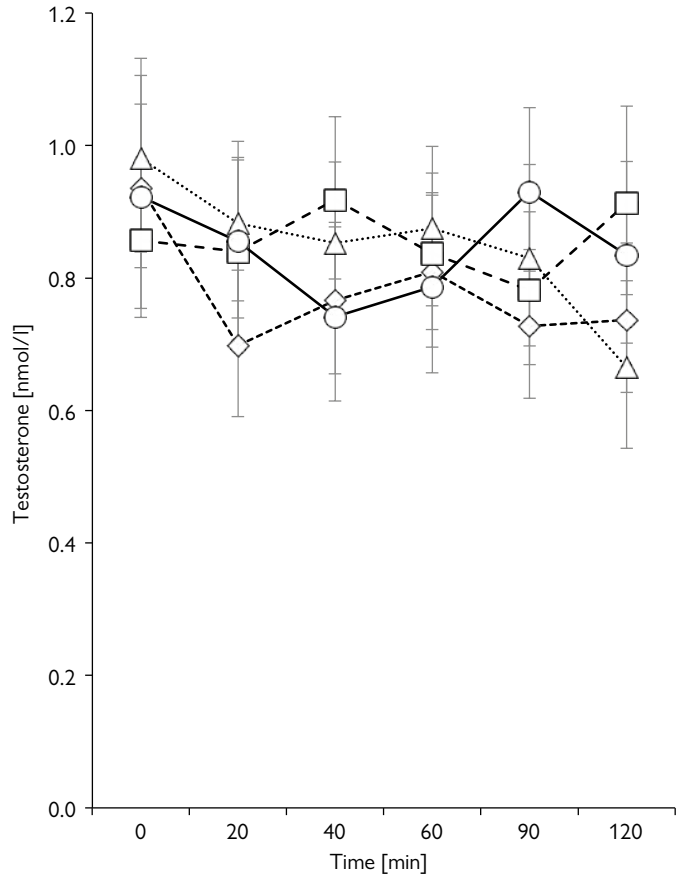

Figure 6 – Testosterone levels after individual stimuli.
Δ – standard breakfast; □ – IVGTT; ◊ – OGTT; ○ – psyllium

further known that OGTT leads to a "blunting" in the character of cortisol decline (Reynolds et al., 2001). This could be explained by the plateau we found in cortisol levels. We found only one paper in the literature showing that psyllium influences corticoid supplies, describing adrenal crisis in a patient treated with prednisone and concurrently given psyllium (Ahi et al., 2011). Oltmanns et al. (2006) studied patients with diabetes mellitus type 2 during 24 hours. They found, that higher cortisol levels where associated with not only higher fasting glucose and glycosuria, but with higher postprandial glycemia, glycosylated hemoglobin, systolic and diastolic blood pressures too. In addition they found (except the morning physiological cortisol surge half hour after arising) an another cortisol peak one hour after the lunch, but after dividing the subject in tertiles they have found, that actually the after-lunch peak of cortisol wasn't present in case of high-cortisol group, in contrast to the other two tertiles. Due to the low number

of participants in our study, we were not able to divide them according to the cortisol levels. But it would be interesting to analyse these founding on non-diabetic subjects.

Giving DHEA for long-term (for 2 years) at a dose of 50 mg/day led to improved glucose tolerance and insulin sensitivity in patients with impaired glucose tolerance (both men and women), and further led to decrease of plasma triglycerides (Weiss et al., 2011). It is also known that moderated alcohol consumption can lead to increased plasmatic DHEAS (Sierksma et al., 2004). However, studies are lacking that would show the effects of food/glucose on DHEA levels. Our results indicate a certain increase of DHEA levels after all stimuli after the initial decline at the beginning of the tests. This increase was most pronounced after intravenous glucose admission, even though the increased levels were delayed compared to the other stimuli despite the fact that the opposite effect might be expected, thanks to the immediate increase of glucose levels after its intravenous application and the highest glycemia obtained from all of the tests.

Our data on the behaviour of melatonin after individual stimuli resulted in interesting findings, which should help to elucidate, for instance, the possibility to influence levels of this hormone through only natural dietary changes. This is important, since melatonin is a hormone of which there is recently much interest and which potentially has a wide spectrum of use. It would be certainly interesting if by intentional control of what we eat or drink, we could influence plasmatic levels of hormones, and in this way obtain their benefits for our health without the need of synthetic hormones or dietary supplements.

Conclusion

By the application of various stimuli we attempted to simulate different situations in the organism, including stimulation through the gastrointestinal (GI) tract as well as bypassing the GI tract by giving glucose intravenously, and by avoiding the caloric effect of food but using mechanical distension of the GI tract through non-caloric fibre. We followed changes in the levels of melatonin and steroid hormones. Melatonin levels physiologically decreased in the morning and a certain increase in levels were found after intravenous glucose (from 20th to 60th minute) and after oral glucose at 60th minute of the tests. The other stimuli did not lead to increased levels. There was a delay in the physiological decline of the cortisol after each individual stimulus (except psyllium), but which was most pronounced after intravenous glucose admission. After the standard breakfast there was an increase in the cortisol level at 40th minute. DHEA levels declined at the beginning of the tests, but then it was followed by a certain increase. One of the weaknesses of our study was the timing of the samplings in the early morning hours, when there are large physiological changes in hormones that might mask small changes induced by the intake of various foods. It would be likely better to perform the study in the later afternoon hours. Another weakness was not including starvation

as a reference profile, even if we used a non-caloric stimulation represented by psyllium. Despite these weaknesses, our results have brought interesting findings.

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Keratocystic Odontogenic Tumour with Extraosseal Spread: Evaluation of the Effect Carnoy's Solution

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Key words: Keratocystic odontogenic tumour – Recurrence – Enucleation – Carnoy's solution

Abstract: Keratocystic odontogenic tumour is relatively rare benign tumour. It is characterized by its fast aggressive growth and high risk of recurrence. Treatment is always surgical: conservative (enucleation, marsupialization) or aggressive (enucleation followed by application of Carnoy's solution, cryotherapy; peripheral ostectomy or en block resection of the jaw). Authors analysed retrospectively 22 patients who fulfilled inclusion criteria, i.e. had odontogenic keratocystic tumour of mandible, wherein antero-posterior dimension was at least 30 mm, and the tumour penetrated into the surrounding soft tissues. All patients underwent tumour enucleation, in 11 patients Carnoy's solution was given into the bone cavity after enucleation. The recurrence rate in the evaluation at least 36 months after surgery was both patient groups the same: 45.4%.

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Introduction

Keratocystic odontogenic tumour (KCOT, previously published under the name of odontogenic keratocysts, first described by Philipsen in 1956) is relatively rare benign tumour characterized by its fast aggressive growth and high risk of recurrence (Ahlfors et al., 1984; Agaram et al., 2004; Madras and Lapointe, 2008).

KCOT incidence is highest among 20–30 years of age (40–60% of all cases of KCOT) (Forssell et al., 1988; Maera et al., 1998; Myoung et al., 2001; Marx and Stern, 2003; Morgan et al., 2005; Madras and Lapointe, 2008), is more common in men than in women. In children less than ten years the occurrence is reduced mostly to association with Gorlin-Goltz syndrome (NBCCS – naevus basal cell carcinoma syndrome) (Ghali and Connor, 2003). Prevalence is more than twice as high in the lower jaw than in maxilla, while the most often affected area is the angle of the mandible and the third molar region with spreading to the body and branch of the jaw (Forssell et al., 1988; Maera et al., 1998; Myoung et al., 2001; Marx and Stern, 2003; Morgan et al., 2005; Madras and Lapointe, 2008). In maxilla the mostly affected area is third molars and incisors area (Voorsmit et al., 1981; Maera et al., 1998; Myoung et al., 2001; Marx and Stern, 2003). KCOT occurrence is solid or multiple, the diameter varies among millimetre to large tumours affecting more anatomic structures (Ghali and Connor, 2003).

KCOT develops from dental lamina epithelium, bazal cells of oral epithelium or the epithelium of enamel organ. Histologically is characterized by presence of keratin in the liquid inside the tumour. Tumour lining is formed by regular squamous epithelium consisting of hyperchromatic cuboidal bazal cells with palisade shaped nuclei (dermoepidermal junction is flat without typical junction naevi). On the surface of the lining is fibrous wall (primarily non-inflamed) with the presence of satellite "daughter" cells (Emerson et al., 1972; Forssell et al., 1988; el-Hajj and Anneroth, 1996; Jordan, 2003; Marx and Stern, 2003).

Most KCOT are clinically asymptomatic (diagnosed commonly as an incidental finding on the radiograph of the jaws). If clinically manifests, it is characterized by the presence of pain and hypoesthesia in area of n. mentalis (caused by pressure on the mandibular canal) and bone expansion. Greater extended tumours may cause looseness of teeth (Ahlfors et al., 1984; Maera et al., 1998; Ghali and Connor, 2003; Marx and Stern, 2003; Madras and Lapointe, 2008). Extensive tumours are prone to rupture. If the rupture occurs, cystic fluid containing keratin penetrates into surrounding soft tissues, which may cause intense inflammatory response accompanied with swelling (Marx and Stern, 2003). In the KCOT localized in maxilla are published cases of tumour expansion to antrum Highmori causing destruction of the orbital floor and eye ball protrusion. Literature reports cases of invasion to skull base (Ahlfors et al., 1984; Gorlin, 1987; Ghali and Connor, 2003).

Diagnostics is based on imaging methods: panoramic and periapical X-ray, computed tomography (CT), magnetic resonance (MR). Characteristic finding is uni- or multilocular cystic lesion. However, the only reliable method of diagnosis is

histological verification of the tumour (Ahlfors et al., 1984; Ghali and Connor, 2003; Marx and Stern, 2003; Madras and Lapointe, 2008).

Treatment of KCOT is surgical. It consists in complete removal of the tumour (Dammer et al., 1997; Bataineh and al Quadah, 1998; Stoelinga, 2001; Ghali and Connor, 2003; Marx and Stern, 2003; Morgan et al., 2005; Maurette et al., 2006).

Retrospective study evaluates recurrence rate 36 months after surgery in patients with KCOT. The presence of recurrence was diagnosed according to the finding of clinical investigation, X-ray, eventually CT scans. Development of recurrence was assessed in relation to the type of surgical treatment: enucleation and enucleation followed by Carnoy's solution (CS) application.

Material and Methods

The retrospective study consists of patients hospitalized and treated surgically with KCOT of the mandible consecutively in the years 2007–2010 at the Department of Dental Medicine, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, Prague, Czech Republic. The study included patients with the ventro-distal dimension of the tumour at least 30 mm. Bone of the jaw was resorbed by the tumour and the tumour was in direct contact with surrounding soft tissues in all cases.

The study included only patients without endocrine, skeletal, rheumatic and oncologic diseases. Patients with Gorlin-Goltz syndrome were excluded. It decreased number of patients to 22.

Study consisted of 22 patients (13 men, 9 women), age range 11–85 years. In all patients the KCOT diagnose was histologically verified, however, all included patients had solidly occurred tumour. All 22 patients underwent enucleation of KCOT under general anaesthesia (in all patients intraoral approach was used). In all cases mechanical curettage of bone cavity followed the enucleation. In 11 cases the curettage was followed by application Carnoy's solution (compound of 3 ml chloroform, 6 ml absolute ethanol, 1 ml glacial acetic acid, 1 g $FeCl_3$; CS). CS was applied on gauze directly in the bone cavity for 60 seconds, once repeated immediately. The procedure was followed by wash out of the cavity with 300 ml sterile saline. CS has been used since 2009 based on literature (Blanas et al., 2000; Madras and Lapointe, 2008; Güler et al., 2012; Johnson et al., 2013), where use of Carnoy's solution after tumour removal decreases recurrence rate. In all cases the surgical wound was closed primarily.

Statistical analysis

Statistical tests suitable for this study were chosen Shapiro-Wilk (S-W) test for normality, Student *t*-test (t) and F-test (F). It is possible to compare both groups (group K, where curettage after tumour removal was performed and group KC, where curettage and subsequently Carnoy's solution application were performed after tumour removing), if we assume that the data are normally distributed and

t-test and F-test indicate equals between these groups. Significance level was p=0.05 for S-W test and p=0.01 for *t*-test and F-test.

Normality test: Shapiro-Wilk normality test
Group K: S-W = 0.9031, p-value = 0.2014
Group KC: S-W = 0.936, p-value = 0.475

We accept hypothesis H_0, as p-value > 0.05 at level of significance p=0.05. Data from both groups (K and KC) are normally distributed. Condition for the other both tests is fulfilled.

Student t-test
Score of this test came out: t = –1.6554, df = 20, p-value = 0.1134. Because p-value is not less than p (p-value > p) at level of significance p=0.01, it is not possible to reject null hypothesis about inequality of both these averages.

F-test
Score of F-test came out: F = 2.7679, num-df = 10, denom-df = 10, p-value = 0.1238. Because p-value is not less than p (p-value > p) at level of significance

Table 1 – Recurrence rate compared to age

Age category (years)	Recurrence (%)		Difference
	C	C+CS	
0–30	0.0%	–	–
31–60	80.0%	37.5%	42.5%
61–90	33.3%	66.6%	–33.3%
Sum (%)			9.2%

C – curettage; C+CS – curettage + Carnoy's solution

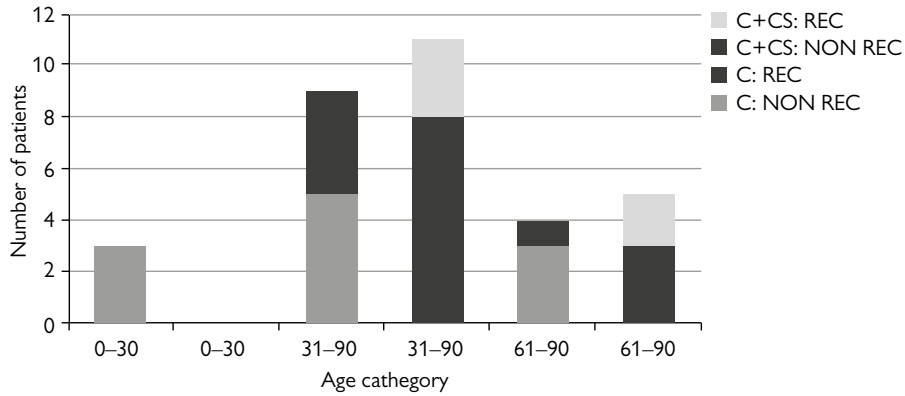

Figure 1 – Age category comparison.

p=0.01, it is not possible to reject null hypothesis about dispersions inequality. There is no significant difference between dispersion in both groups (i.e. selections are from the same basic set with a common variance).

If we assume just absolute number not related to age, the recurrence rate for both groups is the same – 45.4%. When compared to age of patients 3 groups were defined: A1: 0–30 years, A2: 31–60 years, A3: 61–90 years. Recurrence rate review show Table 1 and Figure 1.

- A1: Age group A1 cannot be assessed, because it includes only patients from group C.
- A2: Recurrence rate in group C is 80% (4 from 5 cases). In group C+CS the recurrence rate is 37.5% (3 from 8 cases). In group C+CS the effect of treatment is 42.5% higher than in group C.
- A3: In group C recurrence occur in 33.3% cases (1 from 3 patients). In group C+CS recurrence rate is 66.6% (2 from 3 patients). Group C+CS shows impairment in 33.3% compared to group C.

Results

Distribution by gender

Men – 13 patients (58.3%)
Women – 9 patients (41.7%)

Distribution by age (Figure 2)

The age range of patients was 11–85 years, average age was 45.65 years.

1) decennium (0–10 years) – 0
2) decennium (11–20 years) – 3 patients (1 man, 2 women) – 13.6%
3) decennium (21–30 years) – 0
4) decennium (31–40 years) – 7 patients (5 men, 2 women) – 32.1%
5) decennium (41–50 years) – 3 patients (2 men, 1 woman) – 13.6%
6) decennium (51–60 years) – 3 patients (2 men, 1 woman) – 13.6%

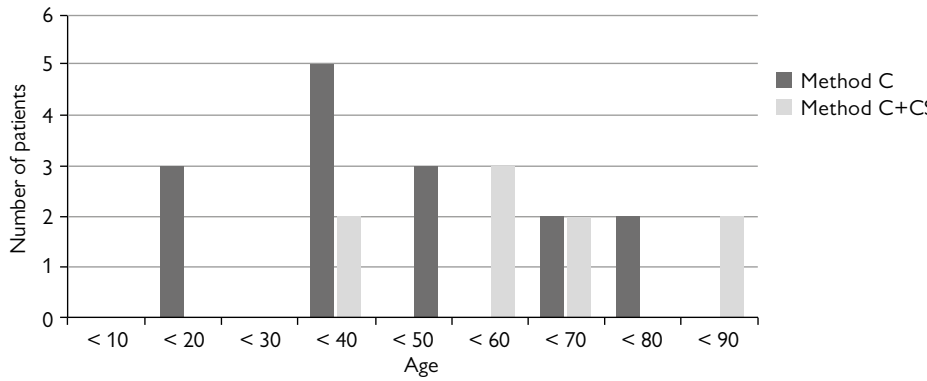

Figure 2 – Distribution of age.

Table 2 – Patients treated with enucleation and curettage

	Type of treatment after enucleation	36 months after operation (recurrence: +)
W, 66 years	C	0
M, 18 years	C	0
M, 35 years	C	0
W, 16 years	C	0
M, 33 years	C	+
M, 32 years	C	+
M, 33 years	C	+
M, 85 years	C	0
W, 62 years	C	+
W, 11 years	C	0
M, 38 years	C	+

W – woman; M – man; C – curettage

Table 3 – Patients treated with enucleation, curettage and CS application

	Type of treatment after enucleation	36 months after operation (recurrence: +)
M, 73 years	C+CS	+
W, 44 years	C+CS	0
W, 32 years	C+CS	0
W, 34 years	C+CS	+
M, 44 years	C+CS	+
M, 57 years	C+CS	0
W, 56 years	C+CS	0
M, 44 years	C+CS	+
M, 67 years	C+CS	0
M, 57 years	C+CS	0
W, 68 years	C+CS	+

W – woman; M – man; C – curettage; C+CS – curettage + Carnoy's solution

7) decennium (61–70 years) – 4 patients (1 men, 3 women) – 18.1%
8) decennium (71–80 years) – 1 patient (man) – 4.5%
9) decennium (81–90 years) – 1 patient (man) – 4.5%

Treatment of bone defect after KCOT removal

Enucleation with curettage – 11 patients (7 men, 4 women). Recurrence rate after enucleation with curettage shows Table 2.

Enucleation with curettage + Carnoy's solution application – 11 patients (6 men, 5 women). Recurrence rate after enucleation with curettage + Carnoy's solution application is shown in Table 3.

Surrounding soft tissues in direct contact with KCOT were not resected.

Table 4 – Age distribution compared to recurrence rate

Age category (years)	C		C+CS	
	non rec	rec	non rec	rec
0–30	3	0	–	–
31–60	5	4	8	3
61–90	3	1	3	2

C – curettage; C+CS – curettage + Carnoy's solution; non rec – no recurrence; rec – recurrence

Complications after Carnoy's solution application

Hypoesthesia, anaesthesia in n. mentalis region ipsilateral to operation site wasn't observed in any patient.

Recurrence rate evaluation

Recurrence number evaluated 36 months after operation was 10 patients (45.4%). In both groups was recurrence rate same – 5 patients. Differences between the two groups in the number of relapses was no significant difference ($p>0.05$) when absolute numbers considered.

Average age in patients group treated with enucleation and curettage was 39.6 years (same for patients with and without presence of recurrence).

In group treated with enucleation and CS application was 52.3 years (in patients with recurrence was 52.6 years) (Table 4).

Adding up – if the assessments for all categories (except A1, which has only been used curettage), reducing the number of relapses by almost 9.2% for the C+CS group compared C.

Discussion

Risks and problems of the treatment of KCOT is a large number of recurrences. In the literature is relapse range 25–60% (Vedtofte and Praetorius, 1979; Maera et al., 1998; Myoung et al., 2001; Marx and Stern, 2003; Maurette et al., 2006; Madras and Lapointe, 2008). In our study the recurrence rate was 45.4%. When evaluating recurrence, it is often difficult to distinguish tumour relapse from bone cavities arising after the extirpation of KCOT. Marx and Stern (2003) indicate the time when it is possible to distinguish this in radiograph, 18 months after surgery. Most recurrences is then described between 5 to 7 year after surgery (Marx and Stern, 2003; Madras and Lapointe, 2008).

There are many theories explaining the reason for a high frequency of recurrences. Generally recognized are:

- Incomplete tumour removal and activation of its growth (tumour does not need any stimulus to activate the growth) (Marx and Stern, 2003). Due to fragility rupture of the tumours wall often occurs. Despite careful curettage of bone

can rest of tumours cells remain in the cavity, activation of the growth causes presence of recurrence (Forssell et al., 1988; Madras and Lapointe, 2008).
- Presence of satellite "daughter" cysts (tumours) developed from the fibrous wall of the removed tumour (Voorsmit et al., 1981; Madras and Lapointe, 2008).
- Formation of new primary KCOT from rest of dental lamina, which are presented as recurrence (Madras and Lapointe, 2008).

Treatment is always surgical, and it is possible to distinguish two types of surgical treatment: conservative and aggressive (Dammer et al., 1997; Maurette et al., 2006; Madras and Lapointe, 2008).

Conservative therapy means:
- Enucleation (with or without) curettage – complete removal of the tumour (eventually followed by curettage of the bone cavity) (Vedtofte and Praetorius, 1979; Jensen et al., 1988; Maurette et al., 2006).
- Marsupialization – resection of vestibular part of bone and opening the tumour intraorally (decompression), with delayed enucleation (Brødum and Jensen, 1991; Dammer et al., 1997; Pogrel and Jordan, 2004; Madras and Lapointe, 2008).

Aggressive treatment means:
- Enucleation followed by peripheral ostectomy – complete tumour removal with resection of adjacent bone (Chow, 1998; Pogrel and Jordan, 2004; Madras and Lapointe, 2008).
- Enucleation followed by chemical curettage with Carnoy's solution – Carnoy's solution is applied into the bone cavity after enucleation for 1 minute, once repeated immediately (Chow, 1998; Stoelinga, 2001; Morgan et al., 2005).
- Enucleation followed by cryotherapy – liquid nitrogen (temperature –70 °C) is applied in cavity after enucleation twice for 1 minute (Jensen et al., 1988; el-Hajj and Anneroth, 1996; Schmidt and Pogrel, 2001).
- En bloc resection of the bone with tumour (el-Hajj and Anneroth, 1996; Morgan et al., 2005; Madras and Lapointe, 2008).

Authors evaluate patients, which underwent simple enucleation of KCOT with patients, which underwent enucleation followed by Carnoy's solution enucleation in this study. Carnoy's solution causes superficial necrosis of the bone to a depth of 1.5 mm. This mechanism eliminates remains of tumours cells and satellite microcysts (Hellstein et al., 2007; Madras and Lapointe, 2008). In both groups the recurrence rate was the same: 45.4%. This result does not correlate with other author's results (Voorsmit et al., 1981; Chow, 1998; Stoelinga, 2001), where recurrence rate was significantly lower (0–11%) after enucleation followed by Carnoy's solution application. Morgan et al. (2005) published recurrence rate after application of Carnoy's solution after KCOT enucleation in 50% cases. However the result is influenced by a small number of patients in study (2 patients). Better

effect for reduce development of recurrences has enucleation followed by application of Carnoy's solution and peripheral ostectomy (Chow, 1998; Morgan et al., 2005; Madras and Lapointe, 2008).

Other authors (Voorsmit et al., 1981; Stoelinga, 2001) show, that enucleation followed using Carnoy's solution has significantly lower incidence of relapse than simple tumour enucleation. Results of this study does not support this idea, which may be caused by a relative small set of patients (22 patients) or the fact, that in all cases tumour was penetrating into surrounding tissues. In cases when tumour penetrates into surrounding tissues is complete removal of tumour difficult. Definitive proof of solution effect when penetrates into surrounding tissues is not published. This result in recurrence rate (45.4%) is achieved if only absolute numbers are considered. When evaluating number of recurrences in relation to age groups (0–30, 31–60, 61–90 years), recurrence rate after CS using is reduced (9.2% lower) than in the patients group where the solution was not used.

Stoelinga (2005) recommends removal of tissues adjacent to KCOT when they were in contact with KCOT. In our study was performed enucleation without resection of soft tissues. This fact may be cause of high number of recurrence.

Long-term stable results are obtained by en bloc resection of the jaw (el-Hajj and Anneroth, 1996; Morgan et al., 2005; Madras and Lapointe, 2008). If resection of the jaw is performed, it has to be considered loss of teeth in affected region, loss of the bone (and planning of following reconstruction – i.e. additional surgery) – all facts impairs the patients quality of life.

Whereas authors prefer enucleation with using Carnoy's solution, which may be eventually accompanied by peripheral ostectomy (Chow, 1998; Morgan et al., 2005; Madras and Lapointe, 2008). Generally respected indication for peripheral ostectomy is repeated recurrences after enucleation or extensive multilocular tumour where enucleation itself would lead to breach of continuity of bone (Marx and Stern, 2003; Madras and Lapointe, 2008). The alternative is bone-sparing resection of the jaw for maintaining its continuity (Ghali and Connor, 2003).

The problem of using Carnoy's solution in bone cavity is effect on the alveolar inferior nerve (if denuded or is free in the cavity after procedure). However, from the results of Frerich et al. (1994), Loescher and Robinson (1998), Wolgen (1999) and Hellstein et al. (2007) is not neurotoxic effect of Carnoy's solution permanent (if the application time is to 2 minutes). This confirms the results of our study: hypofunction of inferior alveolar nerve (manifested by hypoesthesia or anesthesia in mental nerve region) was not detected even in one patient.

Conclusion

KCOT is relatively rare benign tumour occurring in facial skeleton, due this fact is necessary to pay attention on every cystic lesion found on X-ray. Risk of KCOT lies in its aggressive and rapid growth and in the high incidence of its recurrence. Aggressive surgical removal of tumour enucleation followed by Carnoy's solution

application (eventually accompanied by peripheral ostectomy) plays major role in treatment. Recurrence rate may be still high, as shown in our study – this number is related to tumours diameter. Removal of extensive tumours is difficult. In tumours penetrating to surrounding soft tissues is high risk of leaving remains of pat of KCOT. Our study has shown the need for radical resection of tissues around KCOT. Carnoy's solution application after enucleation reduces recurrence rate of 9.2% compared to cases where performed simple enucleation was, our hypothesis was proved.

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Snake Envenomation to the Face of a Child – Rare Case

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Abstract: Snakebites are seen in summer season in the southern part of Turkey, including Hatay province. In average of 40 patients with snakebites are admitted to our hospital every year. Viper is the most common venomous snakes in our region. Their hemotoxins and necrotoxins lead to local or systemic tissue damage and is responsible for the mortality and morbidity. In this report, we described a rare pediatric case, a six-year-old boy having been bitten on the left side of his face when he was looking around from their home's balcony. The patient was orotracheally intubated and mechanically ventilated because of airway obstruction due to severe edema. 12 flacon of anti-snake venom, mannitol infusion, fresh frozen plasma, erythrocytes suspension and antibiotherapy were administered to the patient. Seven days after the admission, clinical and laboratory findings were improved and the patient was discharged in a good condition. Snakebites inflicted on face and neck areas may cause rapidly progressive edema in respiratory tract and lead to life-threatening conditions. Therefore early orotracheal intubation is very important to prevent mortality.

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Introduction

It is estimated that there are about 3,000 snake species in the World and fifteen percent of them have been reported to be venomous (Gold et al., 2002). In Turkey, only 16 of the 46 snake species are considered venomous (Baran, 2005). Approximately 40 snakebite cases are admitted to our hospital every year especially in summer seasons, when snakes and victims are more active. *Crotalidae* subfamily of the *Viperidae* family venom can be classified into five categories: hemotoxic, neurotoxic, necrotoxic, cardiotoxic and nephrotoxic. The most common venomous snake species, *Vipera* is present in the southern area of Turkey, including Hatay province (Baran, 2005). They both have hemotoxic and necrotoxic venoms. Snake venoms have various effects on the bitten site and on the whole body. It may presented with pain, edema, echymosis, lymphangitis, hemorrhagic bullae including puncture wounds at the bitten site, and life-threatening disorders such as renal failure, shock, bleeding and coagulation disorders (Juckett and Hancox, 2002). The intoxication degree is estimated according to the appearance of these symptoms: 1) no intoxication("dry" bite); 2) mild intoxication (local edema and pain); 3) moderate intoxication (pain, edema spreading out of the bite zone, and systemic signs); 4) severe intoxication (shock, severe coagulopathy and massive edemas) (Adukauskiene et al., 2011). The treatment of snakebites consists of supportive therapy (primary wound care, tetanus prophylaxis, antibiotherapy and elevation of the bitten site), administration of antivenom and treatment of complications (coagulopathy and surgical debridement of necrotic zone) (Khimani et al., 2013). This report describes a pediatric case – boy having been bitten on the left side of his face by a venomous snake from *Viperidae* family.

Case report

A six-year-old boy was admitted to our emergency department two hour after having been bitten on the left side of his face by a venomous snake when he was looking around in their home's balcony. Initial clinical examination revealed a confused patient with severe pitting edema starting in the left half of his face and spreading towards neck (Figure 1). Patient's tongue and uvula were also edematic and inspiratory stridor was present. Pulse O_2 saturations was 88%. The patient was orotracheally intubated with a small-sized (4.5 F) endotracheal tube in the emergency department after atropine 0.2 mg, midazolam 2 mg, and vecuronium 4 mg were administered intravenously and mechanical ventilation was initiated. Blood pressure was 70/50 mm Hg, heart rate was 145 beats per minute and sinus tachycardia rhythm was seen in the electrocardiography (ECG) examination. Respiratory rate was 36 breaths/min and auscultation of the lung fields was normal. Laboratory investigation results at the time of admission included a serum glucose level of 136 mg/dl, in blood gas analyses, pH 7.18; HCO_3 16.4; pCO_2 44 mm Hg; pO_2 76 mm Hg; and white blood cell count 10 300/microliter; platelet count 189 000/microliter; and other laboratory findings present in Table 1. Other serum

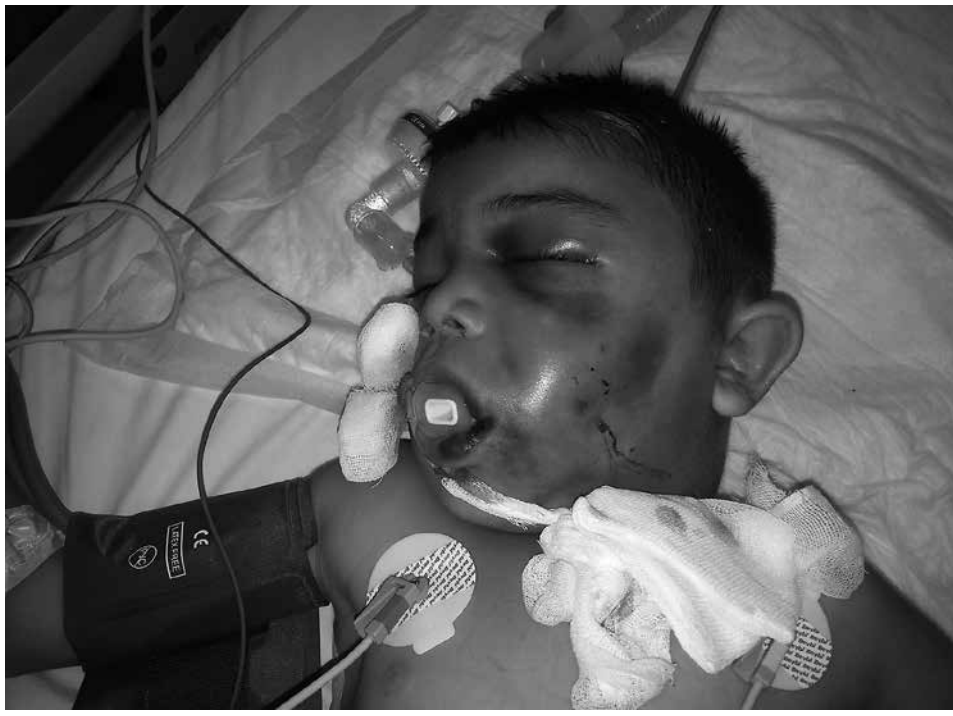

Figure 1 – Initial clinical examination revealed a confused patient with severe pitting edema starting in the left half of his face and spreading towards neck.

chemistry results were within normal limits. Evaluating local and systemic clinical findings together with laboratory test results, the patient was considered to be grade 4, severe envenomation. Following treatment was given as follows; fluid replacement therapy with isotonic normal saline solution (0.9%) was given at a dose of 20 cc/kg in 20 min and 4 flacon snake antivenom/250 cc isotonic saline in one hour (equine, European Institute of Immunology, Zagreb, Croatia), intravenous amoxicilline-clavulanic acid for antibiotherapy (40 mg/kg) and mannitol was given at 1 g/kg for 40 min in the other arm. The patient was re-evaluated after forth hour of treatment. Because no improvement occurred in patient's edema, the mannitol (20%, 4×1 g/kg) and antivenom treatment (4 flacon) infused over one hour and the patient was transported to the intensive care unit. The patient was still orotracheally intubated after the second day of admission. Coagulation parameters and hemoglobin values were not within normal limits (Table 1), thus fresh frozen plasma (FFP) at a dose of 15 cc/kg in one hour and erythrocytes suspension (15 cc/kg) were administered. Additionally, 3×2 flacons (a total of 6 grams) of antivenom were administered. After the treatment, patient's laboratory findings were normalized (Table 1). The third day of the admission, edema was regressed,

Table 1 – Laboratory findings

Day	Hb (g/dl)	Htc (%)	PT (s)	PTT (s)
1.	9.2	27.8	21.1	49.5
2.	7.7	21.5	25.5	78.2
3.	9.6	28.0	16.7	23.0
7.	10.5	31.5	14.5	22.0

Hb – hemoglobin; Htc – hematocrit; PT – prothrombin time; PTT – partial thromboplastin time

antivenom administration was not given and the patient was extubated after remaining in T-tube for 8 hours. The mannitol dosage was gradually decreased and stopped and the patient was transported to the normal service for antibiotherapy and fluid replacement therapy. Seven days after admission, the patient was discharged in a good condition (Figure 2).

Discussion

After a venomous snakebite, the venom is activated by body temperature and tissue pH. Crotaline venoms are heterogeneous enzymes that cause 3 types of toxicity. Most obvious is local tissue cytotoxicity manifested as edema and necrosis

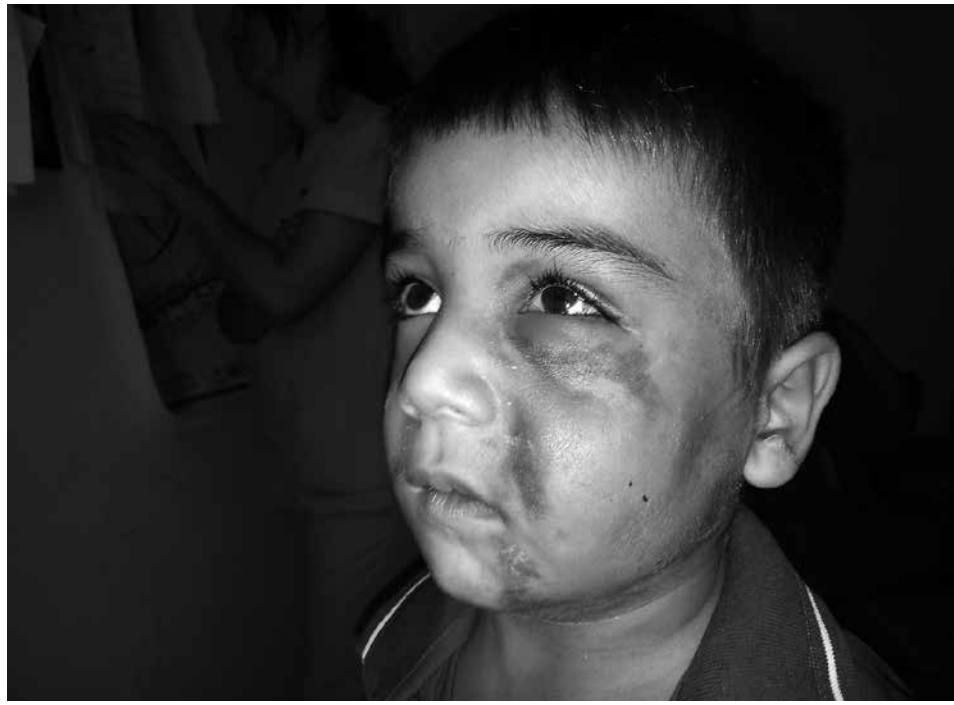

Figure 2 – Seven days after admission, the patient was discharged in a good condition.

caused by direct cellular injury from venom components. Thrombocytopenia and coagulopathy, similar in appearance to disseminated intravascular coagulation, are typical hematologic findings. In certain rattlesnake species, neurotoxic symptoms may predominate. Ptosis, fasciculations, weakness, and eventually, paralysis may occur. The envenomation by *Crotalidae* species leads to local swelling in bitten site within 15–30 minutes but it may be longer (one to two days) according to severity of the case. In severe cases, edema can involve an entire limb within an hour. In addition to edema, life-threatening bleedings and coagulation disorders, renal failure and shock can be seen (Juckett and Hancox, 2002).

Traditional management of snake bites consists of aggressive supportive care (cleaning the wounds, fluid replacement, analgesia, tetanus toxoid, antibiotic therapy, extremity immobilizing – elevating) and antivenom therapy that is the mainstay of treatment (Gold et al., 2002). The factors in antivenom therapy are the size of edema, coagulopathy, shock or other systemic disorders. Thus, it is important to measure the border of edema in every 30 minutes. In moderate and severe envenomation, anti-snake venom should also be administered within 4 hours after snakebite but can still be effective within 24 hours. Antivenom neutralizes circulating venom, eliminating the toxic effects and thus effectively corrects clinical and laboratory signs; however, they are not necessarily effective as far as thrombocytopenia and rhabdomyolysis are concerned (Nuchpraryoon and Garner, 2000; Agency for Clinical Innovation, 2007; Kliegman et al., 2007; Norris, 2008; Warrell, 2010). Anti-snake venom is made by immunizing large animals, like horses, with venom or multiple venoms that often (< 10%) may cause acute hypersensitivity reactions because of their protein nature (Norris, 2008).

Fasciotomy is not routinely recommended but compartment pressure should be monitored closely. If it exceeds 30 mm Hg, fasciotomy should be performed. Facial *Crotalidae* envenomation is rarely reported (Pfeiffer and Price, 1976; Gerkin et al., 1987; Lewis and Portera, 1994; Tanen et al., 2001).

The vast majority of *Crotalidae* bites is located on the extremities that limbs below elbows or knees are the most commonly bitten body area (90%), with less than 2% to the head or neck (Gerkin et al., 1987; Adukauskiene et al., 2011). A few cases of snake bites from face have been reported in the literature and most of them in adults (Pfeiffer and Price, 1976; Gerkin et al., 1987; Lewis and Portera, 1994; Tanen et al., 2001).

In a case of facial bite, nasotracheal intubation was required and antivenom was initiated but discontinued when allergic symptoms began despite treatment with methylprednisolone and triamcinolone. After 5 days of supportive care, the patient was discharged home without sequelae (Pfeiffer and Price, 1976). In a case of snake bites from tongues, patient required nasotracheal intubation, 35 total flacons of polyvalent antivenom, and was safely extubated 5 days after admission (Gerkin et al., 1987). In another case, patient was envenomated in the left cheek and nasotracheally intubated and received antivenom along with supportive care

(Lewis and Portera, 1994). A 14-month-old female toddler was envenomated by *Crotalus viridishelleri* above the right upper lip. Oropharyngeal edema necessitated emergent orotracheal intubation. A total of 16 flacons of antivenom were administered over 24 hours. The patient was extubated 5 days later (Richardson et al., 2005). Nevertheless, a 22-year-old man of *Vipera berus* snake bite to the forehead was reported. In this case, antivenom was not given because of high allergy risk. There was a persistent unilateral facial frontalis muscle paresis (Weinelt et al., 2002).

In our case, the child was bitten on the left side of its face by a venomous snake when he was looking around in their home's balcony. Two hours after having been bitten, respiratory distress developed due to severe swelling in uvula, face and neck areas (Figure 1). Aggressive snake antivenom administration and supportive care are fundamental interventions necessary to prevent snake envenomation-induced morbidity and mortality. In contrast to our case, antivenom was not given in the case of Weinelt et al. (2002) and persistent unilateral facial frontalis muscle paresis developed subsequently (Richardson et al., 2005). In other cases, despite the antivenom therapy, patients were able to be extubated after at least 5 days and mannitol was not given in these cases (Lewis and Portera, 1994; Weinelt et al., 2002). In another study, mannitol was used as monotherapy and successful results were obtained (Anil et al., 2011). Therefore early orotracheal intubation was performed and mannitol infusion was started to decrease airway tract edema at the same time together with antivenom administration in our case. The patient was extubated on third day. We think that mannitol may reduce the duration of orotracheal intubation by decreasing the airway edema.

Conclusion

Here a rare case of a pediatric patient with a rattlesnake envenomation to the face that required emergent orotracheal intubation to prevent airway obstruction is presented. We think that early airway protection, intravenous crotaline Fab antivenom therapy, and simultaneous mannitol administration is a successful combination in this case.

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