Bulgarian Journal of Veterinary Medicine, 2015, **18**, No 3, 194–208
ISSN 1311-1477; DOI: 10.15547/bjvm.886

Original article

EFFECT OF ANTIOXIDANT TREATMENT ON SOME INDICATORS OF OBESITY-INDUCED CHANGES IN INSULIN SENSITIVITY AND BETA-CELL FUNCTION IN NEW ZEALAND WHITE RABBITS

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Summary

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The current study was conducted to investigate the impact of dietary antioxidant supplementation on obesity-induced changes in some surrogate indices of insulin sensitivity and ß-cell function in New Zealand white rabbits. Three groups of rabbits were used in this experiment: castrated animals treated with antioxidants (vitamin E and d-limonene, Immunoprotect) (Cim; n=6), castrated obese animals (CO; n=6) and non-castrated non-obese controls (NC; n=7). At the end of the follow-up period of 2 months after castration an intravenous glucose tolerance test (IVGTT) was performed after 12-hour fasting. Blood samples for determination of simplified estimates of insulin resistance and β-cell function were obtained at baseline and at various time intervals over the 120-min test. In addition, lipid content in *m. Longissimus lumborum* и *m. Semimembranosus* was determined. Some of the simplified measurements of insulin resistance (fasting insulin, fasting insulin to glucose ratio, $HOMA_{ins.resist}$ index), beta-cell function ($HOMA_{\beta\text{-cell}}$, $AUC_{insulin\ 0\rightarrow 60\ min}$) and muscles lipid content in CO were higher while QUICKI and Bennett indices were lower than in controls. No differences in surrogate indices between CIm and NC groups were found suggesting improvement of insulin sensitivity and β-cell function after antioxidant supplementation. Surrogate indices are simple and reliable indicators of insulin sensitivity and β-cell function in rabbits as they were closely associated with markers of obesity and can be modified by antioxidant supplementation.

Key words: antioxidant supplementation, insulin resistance and β-cell function indices, obesity, rabbits

INTRODUCTION

Decreased insulin sensitivity, termed insulin resistance (IR), can in part be defined as a reduced ability of insulin to activate glucose uptake and intracellular metabolism in target tissue (Saltiel, 2001; Gerich, 2003; Kahn, 2003; Weiss, 2007; Dimitrova & Georgiev, 2007). Reduced insulin action in skeletal muscle, is the primary defect responsible for the development of hyperglycaemia which is the hallmark of the impaired glucose tolerance and type 2 diabetes (Henriksen & Dokken, 2006; Corcoran *et al.*, 2007; DeFronzo & Tripathy, 2009; Samuel *et al.*, 2010). Despite extensive investigations, showing inverse association between the increased intramuscular lipid content and insulin sensitivity, the underlying molecular underpinnings of lipid-induced insulin resistance in skeletal muscle remains unclear (Morino *et al.*, 2006; Haugaard *et al.*, 2009; Henriksen, 2010; Muoio, 2010; Martins *et al.*, 2012).

An emerging body of evidence revealed the importance of defects in β-cell function and insulin secretion for the impairment of glucose homeostasis in obesity and/or type 2 diabetes (Festa *et al.*, 2008; Poitout & Robertson, 2008; Giacca *et al.*, 2010). Although it is generally accepted that the risk of developing diabetes is determined by the combination of genetic susceptibility of the β-cells and damaging effects of hyperglycaemia and/or hyperlipidaemia, termed glucolipotoxicity, the mechanisms involved in β-cell failure are largely unknown (Lu *et al.*, 2010).

Several insulin sensitivity indices – homeostasis model assessment ($HOMA_{ins.res.}$), quantitative insulin sensitivity check index (QUICKI), insulin sensitivity index (ISI) and β-cell function – $HOMA_{β\text{-}cell}$, insulinogenic index, acute phase of insulin secretion (AIR) are used in humans (Tripathy *et al.*, 2004; Chen *et al.*, 2005; Festa *et al.*, 2008; Martinez-Hervas *et al.*, 2011), dogs (Irvine *et al.*, 2002; Larson *et al.*, 2003) and cats (Appleton *et al.*, 2005) but so far not in rabbits. At the same time, many features of lipoprotein metabolism in rabbits are similar to those in humans (so-called LDL mammals) but differ from the most widely used experimental animals – rats and mice, which are predominantly HDL animals (Kitajima *et al.*, 2004; Zheng *et al.*, 2009; Waqar *et al.*, 2010). That is why rabbits are increasingly used as appropriate animal models to study pathogenic mechanisms of obesity-associated abnormalities in lipid and glucose metabolism such as insulin resistance, dyslipidaemia, atherosclerosis, metabolic syndrome and type 2 diabetes (Kainuma *et al.*, 2006; Kawai *et al.*, 2006; Zhao *et al.*, 2007; Waqar *et al.*, 2010). Therefore, there is an increasing need to apply simple and reliable markers for evaluation of insulin sensitivity and β-cell function in rabbits.

Obesity has been described as a state of chronic inflammation, associated with oxidative stress which seems to play an important pathogenic role in the vicious cycle linking obesity, insulin resistance and type 2 diabetes (Franzini *et al.*, 2008; Roberts & Sindhu, 2009; Valdecantos *et al.*, 2009; Lin *et al.*, 2012). Furthermore, reactive oxygen species (ROS) have been defined as an initial key factor triggering obesity-induced insulin resistance as their increased generation preceded the elevation of tumour necrosis factor-alpha (TNF-α) and free fatty acids (FFA) in the plasma and liver (Matsuzawa-Nagata *et al.*, 2008). However, despite the well documented negative impact of ROS on insulin action and insulin secretion, the evidence for a

beneficial effect of exogenous antioxidants is inconsistent because many interventional studies yield conflicting results. (Davis *et al.*, 2002; Houstis *et al.*, 2006; Franzini *et al.*, 2008; Singh *et al.*, 2008; Bashan *et al.*, 2009). In addition, the impact of antioxidants on insulin resistance and β-cell dysfunction in most trials is too weak, hence, further studies are required in order to better understand the effects of dietary antioxidants on obesity-associated disorders (Opara, 2004; Bashan *et al.*, 2009; Boudina *et al.*, 2012; Garcia-Diaz *et al.*, 2012).

Recently, our laboratory created an animal model, using castrated male New Zealand white rabbits and reported that 2 months after castration rabbits developed visceral obesity, dyslipidaemia and impaired glucose tolerance (Georgiev *et al.*, 2011). Antioxidant (vitamin E and d-limonene) treatments affected favourably blood lipid profile and glucose kinetics (Georgiev *et al.*, 2009; 2011). In addition, we established reference ranges of some glucose kinetic parameters during intravenous glucose tolerance test (IVGTT) in lean rabbits (Dimitrova *et al.*, 2008).

The current study was conducted to investigate the impact of dietary antioxidant supplementation on the obesity-induced changes in some indices of insulin sensitivity and ß-cell function in New Zealand white rabbits.

MATERIALS AND METHODS

Experimental design and experimental procedures

The model of visceral obesity was created by castration of male New Zealand white rabbits as previously described (Georgiev *et al.*, 2009; 2011). The experimental procedure was approved by the Commission of Ethics at the Faculty of Veterinary Medicine of Trakia University, Stara Zagora. During the entire experimental period the recommendations for caring and treatment of rabbits reared as experimental animals were followed.

The design of the experiment has already been reported in details (Georgiev *et al.*, 2011). Briefly, 2–2.5 month-old rabbits were randomly divided into 3 groups: i) first group (CIm; n=6) – castrated obese and treated with Immunoprotect for 2 months; ii) second group (CO; n=6) – castrated obese; and iii) third group (NC; n=7) – control group, non-castrated non-obese. Imunoprotect is an oily nutritional supplement. It contains two antioxidants: vitamin E (10 mg equivalent to 15 IU) and organic extract from citrus fruits peel (90 mg), which contains in high proportion d-limonene. Imunoprotect was produced and provided by Pharmaray, Sofia, Bulgaria as gelatinous capsules. The rabbits from the first group received two capsules *per os* daily before the morning feeding for 2 months.

At the end of the follow-up 2-month period after castration an intravenous glucose tolerance test (IVGTT) was performed after 12-hour fasting (Liu *et al.*, 2005; Zhao *et al.*, 2007; Georgiev *et al.*, 2011). Blood samples for determination of simplified measurements of insulin resistance and β-cell function were obtained at baseline and at various time over the 120-min duration of the test.

Blood samples for the determination of insulin and glucose concentrations were collected from the jugular veins. Heparin was used as anticoagulant to obtain plasma for the insulin assay. The blood samples were centrifuged immediately after the collection at 800×*g* for 15 min. The plasma was stored in plastic tubes at –20 ^{0}C until assayed. Glucose concentration was measured in whole blood.

Laboratory methods

Plasma insulin concentration was measured by radioimmunoassay with a commercially available kit adapted for rabbits (Immunotech, Prague, Czech Republic). The blood glucose concentration was measured immediately after collection of the samples with a glucosemeter (Home Diagnostics, Inc., Ft. Lauderdale, Florida, USA) based on glucose oxidase method using one drop of whole blood.

Lipid content in muscle samples was determined after extraction with chloroform/methanol as described (Gondret *et al.*, 2004). Muscle lipid content was expressed as g per 100 g fresh tissue.

Indices of insulin sensitivity

The surrogate indices of insulin sensitivity were calculated as described in cats (Appleton *et al.*, 2005). The following parameters were determined: fasting insulin (Io), fasting insulin to glucose (Go) ratio (Io/Go), insulin concentration at 60^{th} and 120^{th} min ($I_{60\ min}$ and $I_{120\ min}$) and insulin to glucose ratio at 60^{th} and 120^{th} min. after glucose infusion.

Some simplified insulin sensitivity estimates ($HOMA_{ins.\ res.}$, QUICKI and the Bennett index) were calculated from insulin and glucose values at baseline using the following equations:

1) $HOMA_{ins.\ res.} = (I_0 \times G_0)/22.56$ (Mattheeuws *et al.*, 1984; Wallace *et al.*, 2004; Chen *et al.*, 2005);

2) $QUICKI = 1/[\log I_0 + \log G_0]$ (Wallace *et al.*, 2004; Appleton *et al.*, 2005; Chen *et al.*, 2005);

3) Bennett index $= 1/\log I_0 \times \log G_0$ (Appleton *et al.*, 2005; Ciampelli *et al.*, 2005);

where: I_0 is the fasting insulin (μU/mL) and G_0 is the fasting glucose (mmol/L). Higher $HOMA_{ins.\ res.}$ and lower QUICKI and Bennett indices are indicators of increased insulin resistance.

Indices of ß-cell function

$HOMA_{\beta\text{-cell}}$ was calculated using the equation:

$HOMA_{\beta\text{-cell}} = (20 \times I_0)/(G_0 - 3.5)$ (Mattheeuws *et al.*, 1984; Wallace *et al.*, 2004).

The indices characterising the first or early phase of insulin secretion and insulin secretion during the 1^{st} and 2^{nd} hours after glucose loading during IVGTT were calculated as shown in dogs (Irvine *et al.*, 2002; Larson *et al.*, 2003; Slavov *et al.*, 2010). The highest values of insulin and glucose were considered peak values and the increments of insulin and glucose concentration above their respective fasting levels were considered as ΔI and ΔG (Larson *et al.*, 2003). Early phase insulin secretion in response to glucose infusion was calculated as the insulinogenic index (ΔI/ΔG), the area under the curve for insulin was determined from 0 to 10 min ($AUC_{insulin\ 0\rightarrow10\ min}$) and insulin to glucose ratio – by the 10^{th} min ($I_{10min}/G_{10\ min}$) (Larson *et al.*, 2003). Insulin secretions during the 1^{st} and 2^{nd} hour after IVGTT were calculated as $AUC_{insulin\ 0\rightarrow60\ min}$ and $AUC_{insulin\ 60\rightarrow120\ min}$, respectively (Larson *et al.*, 2003; Slavov *et al.*, 2010).

Histological examination

At the end of the experiment (2 months after castration) 3 rabbits from each group were sacrificed by an overdose of thiopental sodium. Material for histological examination was taken from *m. Longissimus lumborum* (LL). The obtained tissue specimens were fixed in Bouin's fixative and in 10% neutral-buffered formalin. After fixation the tissue specimens were embedded in paraffin, cut into 6 μm thick sections and stained with

haematoxylin and eosin. In addition, tissue samples for determination of lipid content were obtained from *m. Longissimus lumborum* and *m. Semimembranosus* (SMB). The results for markers of obesity (body weight – BW, body mass index – BMI, amount of visceral fat), lipid profile (plasma triglycerides, total cholesterol, high- and low-density lipoprotein cholesterol concentrations) and some IVGTT parameters ($K_{el\ glucose}$, $AUC_{glucose\ 0\rightarrow120\ min}$, $AUC_{insulin\ 0\rightarrow120\ min}$ and $AUC_{insulin\ 0\rightarrow120\ min}/AUC_{glucose\ 0\rightarrow120\ min}$ ratio) are published elsewhere (Georgiev *et al.*, 2011).

Statistical analysis

Statistical analyses were performed using Statistica v.6.1 for Windows (StatSoft Ins., USA, 1984-2002). All data are presented as means ± standard error of the mean (mean ± SEM) calculated according to standard descriptive statistical tests. The ANOVA was used to evaluate the significance of the differences in the quantitative variables (concentrations of insulin, surrogate indices of insulin resistance and β-cell function and skeletal muscle lipid and glycogen contents) between the three experimental animal groups. When the group effect was significant, the differences between groups were determined by the *post hoc* LSD test. Correlations between markers of obesity and calculated parameters of insulin sensitivity and β-cell function were determined, using the Pearson correlation analysis at a level of significance $P<0.05$.

RESULTS

Indices of insulin sensitivity

The mean values of insulin sensitivity indices are presented in Table 1. Basal insulin concentrations and their respective insulin to glucose ratios in CO were significantly ($P<0.01$) higher than in CIm and control (NC) groups. Insulin concentrations at min 120 did not differ between groups while insulin concentration at min 60 in CO was significantly ($P<0.05$) higher than in CIm. Mean insulin to

Table 1. Surrogate indices of insulin sensitivity (Mean±SEM) in the three groups of rabbits: castrated, obese, and treated with Immunoprotect (CIm group; n=6); castrated-obese (CO group; n=6) and non-castrated, non-obese (control) rabbits (NC group; n=7)

Indices of insulin sensitivity	Abbreviations	CIm group	CO group	NC group
Basal insulin (μU/mL)*	I_0	1.70 ± 0.25^b	11.0 ± 2.73^a	2.90 ± 0.94^b
Basal insulin to glucose ratio*	I_0/G_0	0.34 ± 0.06^b	2.20 ± 0.44^a	0.74 ± 0.26^b
Insulin after glucose injection (μU/mL)*	I_{60min}	1.80 ± 0.65^b	7.80 ± 3.06^a	3.40 ± 1.26
	I_{120min}	1.30 ± 0.37	5.80 ± 2.98	2.80 ± 0.48
Insulin/glucose ratio after glucose injection*	$I_{60\ min}/G_{60\ min}$	0.29 ± 0.9	1.67 ± 0.86	0.80 ± 0.31
	$I_{120\ min}/G_{120\ min}$	0.21 ± 0.42	1.39 ± 0.81	0.58 ± 0.09
$HOMA_{ins.\ resist.\ index}$*		0.40 ± 0.08^b	2.16 ± 0.69^a	0.48 ± 0.1^b
QUICKI index**		1.24 ± 0.23^b	0.73 ± 0.13^a	1.23 ± 0.19^b
Bennett index**		7.04 ± 1.59^b	1.62 ± 0.26^a	4.98 ± 1.37^b

*The higher the value, the lower the insulin sensitivity; **The lower the value, the lower the insulin sensitivity. Means with different superscripts within the same row differ at $P<0.05$.

Table 2. Surrogate indices of beta-cell function (Mean±SEM) in the three groups of rabbits: castrated, obese, and treated with Immunoprotect (CIm group; n=6); castrated-obese (CO group; n=6) and non-castrated, non-obese (control) rabbits (NC group; n=7)

Indices of beta-cell function	Abbreviations	CIm group	CO group	NC group
Insulinogenic index	$\Delta I/\Delta G$	4.9 ±1.8	5.6 ± 2.73	5.31 ± 0.49
Insulin to glucose ratio	I_{10min}/G_{10min}	3.6 ± 1.35	4.5 ± 1.8	3.9 ± 0.42
HOMA β cell		39.8 ± 16.9[b]	153.7 ± 32.2[a]	29.3 ± 7.2[b]
Area under the insulin curve 0→10min (μU/mL×min)	$AUC_{ins\ 0\rightarrow10min}$	149.3 ± 49	210.9 ± 71	145.5 ± 17
Area under the insulin curve 0→60 min (μU/mL×min)	$AUC_{ins\ 0\rightarrow60min}$	106.0 ± 22[b]	564.0 ± 118[a]	190.3 ± 57[b]
Area under the insulin curve 60→120 min (μU/mL×min)	$AUC_{ins\ 60\rightarrow120min}$	95.0 ± 30	407.5 ± 174	201.0 ± 51

Means with different superscripts within the same row differ at P<0.05.

glucose ratios at min 60 and 120 in CO rabbits tended to be higher (P<0.1) than those in the Cim groups, while there were no differences between both groups of castrated rabbits (CO and CIm) vs control group (NC). $HOMA_{ins.resist}$ values in CO were significantly (P<0.01) higher than both control (NC) and CIm groups. In contrast, QUICKI and Bennett indices in CO were substantially (P<0.05) lower than in control and Cim rabbits. Meanwhile, no differences in $HOMA_{ins.resist.}$, QUICKI and Bennett indices were found between control rabbits and those treated with Immunoprotect (Table 1).

Indices of ß-cell function

The mean values of beta-cell function indices are presented in Table 2.

$HOMA_{\beta\ cell}$ index in CO was significantly (P<0.001) higher than in NC and CIm. There were no differences in the indices, characterising the first (early) phase of insulin secretion in response to exogenous glucose loading during IVGTT ($\Delta I/\Delta G$, I_{10min}/G_{10min} and $AUC_{insulin\ 0\rightarrow10min}$). Insulin secretion during the 1st hour after glucose infusion ($AUC_{insulin\ 0\rightarrow60\ min}$) in CO was considerably (P<0.01) higher than in NC and Cim groups. During the 2nd hour after glucose infusion ($AUC_{insulin\ 60\rightarrow120\ min}$) insulin secretion in CO was also higher than in NC and CIm although not significantly.

Chemical composition of skeletal muscles

Mean concentrations of lipids in *m. Longissimis lumborum* (LL) and *m. Semimembranosus* (SBM) are presented on Fig. 1. Lipid SMB content in non-castrated non-obese rabbits (controls) tended to be higher than that of LL, while two months after castration it increased more markedly in LL muscle (Fig. 1). Lipid content of LL muscle in castrated obese rabbits was significantly greater than both controls (P<0.001) and castrated, obese animals supplemented with

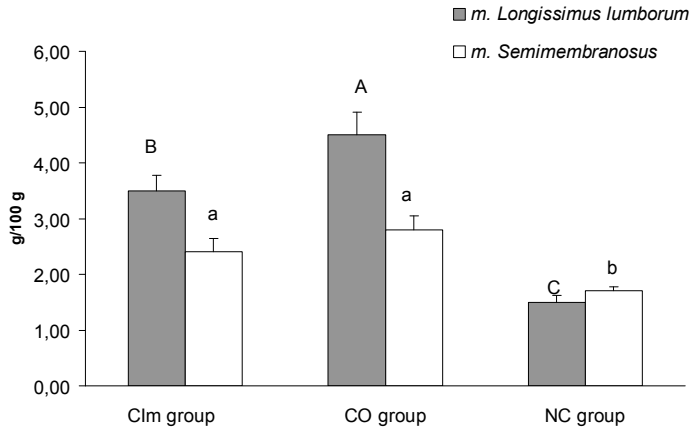

Fig. 1. Lipid content (g/100 g) of *m. Longissimus lumborum* and *m. Semimembranosus*in castrated obese rabbits treated with antioxidants (Cim group), castrated obese rabbits (CO group) and non-castrated non-obese rabbits (NC group) Different capital letters show statistically significant differences between groups with respect to m. *Longissimus lumborum*; while different lower case letters – significant differences between groups for m. *Semimembranosus.*

Immunoprotect (P<0.05). Lipid SMB content in castrated rabbits from CO and CIm groups was significantly (P<0.01) higher than in non-castrated non-obese (NC) animals (Fig. 1).

Histological examination

Light microscopy of muscle samples revealed increased fat deposition in LL muscle of rabbits from the CO group (Fig. 2). In the CIm group, a lower fat content in muscle samples was detected, while in non-castrated non-obese (control) rabbits no adipocytes were observed. In all samples, the adipose tissue was predominantly located in the perimysium.

Correlation analysis

Basal insulin correlated significantly positively with $HOMA_{ins.\ resist.}$ (r=0.94; P<0.001), $HOMA_{\beta\ cell}$ (r=0.63; P<0.01), triglycerides (r=0.77; P<0.01) and $AUC_{insulin\ 0\rightarrow60\ min}$ (r=0.66; P<0.01) and tended to correlate with the amount of visceral fat (r=0.41; P<0.1). $HOMA_{ins.\ resist.}$ correlated significantly positively with BW (r=0.55; P<0.05), triglycerides (r=0.73; P<0.01), $AUC_{insulin\ 0\rightarrow60\ min}$ (r=0.81; P<0.01) and $AUC_{insulin\ 60\rightarrow120\ min}$ (r=0.81; P<0.01) and tended to correlate with amount of visceral fat (r=0.40; P<0.1). The Bennett index correlated significantly negatively with basal insulin (r=–0.71; P<0.01), $HOMA_{ins.\ resist.}$ (r=–0.67; P<0.01), triglycerides (r=–0.79; P<0.01), I_0/G_0 (r=–0.72; P<0.01) in *m.* SMB (r=–0.64; P<0.01). I_0/G_0 ratio correlated significantly positively with triglycerides (r=0.80; P<0.01) and $HOMA_{ins.\ resist.}$ (r=0.93; P<0.001). $HOMA_{\beta\ cell}$ correlated significantly positively with triglycerides (r=0.72; P<0.01), $AUC_{insulin\ 0\rightarrow60\ min}$ (r=0.64; P<0.01) in SMB (r=0.65; P<0.01).

Lipid concentration in SMB correlated significantly positively with BW (r=0.48; P<0.05) and visceral fat (r=0.48; P<0.05) and tended to correlate negatively with

$K_{el\ glucose}$ (r=–0.41; P<0.1). Lipid concentration of LL correlated significantly positively with $AUC_{insulin\ 0\rightarrow60\ min}$ (r=0.62; P<0.01) and visceral fat amount (r=0.61; P<0.01).

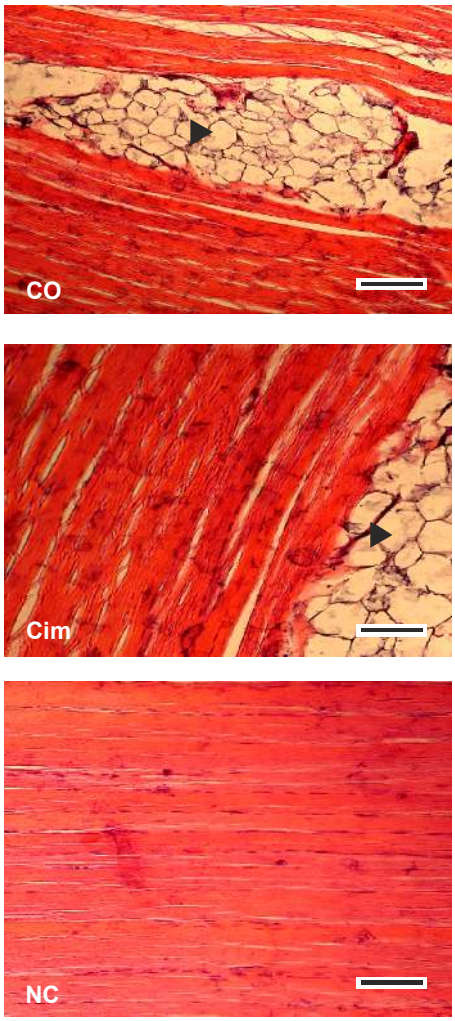

Fig. 2. Longitudinal section of skeletal muscle in castrated obese rabbits (CO), castrated and treated with Immunoprotect rabbits (Cim) and non-castrated rabbits (NC); arrowheads – adipose tissue; H/E; bar=50 μm.

There was a strong positive correlation of insulinogenic index (ΔI/ΔG) with $AUC_{insulin\ 0\rightarrow10\ min}$ (r=0.98; P<0.001) and I_{10min}/G_{10min} (r=0.99; P<0.001).

DISCUSSION

The visceral or central obesity in humans is the main predisposing factor for various metabolic abnormalities such as insulin resistance, metabolic syndrome, type 2 diabetes and cardiovascular diseases (Lewis *et al.*, 2002; Ibrahim, 2010; Galic *et al.*, 2010; Slavov & Dzelebov, 2010). A growing number of studies show that obesity-associated systemic and/or organ-specific oxidative stress is a crucial factor involved in the impairment of insulin sensitivity and ß-cell function (Dokken *et al.*, 2008; Poitout & Robertson, 2008; Bashan *et al.*, 2009; Valdecantos *et al.*, 2009). The use of some simple and reliable indicators of insulin resistance and β-cell function will be advantageous over the "gold standard" hyperinsulinaemic euglycaemic clamp, which is complicated, labour intensive and time demanding procedure (Chen *et al.*, 2005; Muniyappa *et al.*, 2009).

Recently we found that high-fat feeding-induced obesity in dogs is associated with marked impairment of insulin sensitivity and β-cell function (Slavov *et al.*, 2010). In the current study we demonstrated that basal insulin concentration, $HOMA_{ins\ res}$, I_0/G_0 ratio, $HOMA_\beta$ cell, $AUC_{insulin\ 0\text{-}60\ min}$ and intramuscular lipid content in castrated obese rabbits were significantly higher while QUCKI and Bennett indices were significantly lower than in non-castrated non-obese rabbits and correlated with the markers of obesity. Antioxidant supplementation favourably modified surrogate indices of insulin sensitivity and β-cell function.

Our results indicated a marked hyperinsulinaemia at baseline in castrated-obese rabbits which is consistent with other data in obese rabbits (Kawai *et al.*, 2006; Zheng *et al.*, 2009). Obesity-induced fasting hyperinsulinaemia has been also described in cats (Thiess *et al.*, 2004; Appleton *et al.*, 2005), dogs (Verkest *et al.*, 2005; Verkest *et al.*, 2011) and humans (Festa *et al.*, 2008; DeFronzo & Tripathy, 2009; Gil-Campos *et al.*, 2010). In addition, we found significantly higher values of fasting insulin to glucose ratio, $HOMA_{ins.\ resist}$ and $HOMA_{\beta\text{-cell}}$ and significantly lower QUICKI and Bennett indices in obese than in lean rabbits. Similar obesity-induced changes of surrogate insulin estimates were described in cats (Appleton *et al.*, 2005). Basal insulin and $HOMA_{ins.\ resist.}$, were positively correlated with body weight, the amount of intraabdominal fat and plasma triglycerides, showing that hyperinsulinaemia in castrated rabbits was closely associated with visceral obesity. Usually, during the early stage of insulin resistance, β-cell function and insulin secretion are increased to maintain normoglycaemia in the face of decreased sensitivity and/or responsiveness of target tissues to insulin (Bergman *et al.*, 2002; Weir & Bonner-Weir, 2004). This is further confirmed by the significant positive correlation between muscle lipid content and $AUC_{ins\ 0\rightarrow 60\ min.}$

The strong correlation between $HOMA_{ins.\ resist}$ and basal plasma insulin in our study indicates that measurement of plasma insulin in rabbits with food withheld for 12 h could be used in research settings as a reliable predictor of insulin resistance.

In humans, Weir & Bonner-Weir (2004) propose five stages of evolving β-cell dysfunction during progression to diabetes, each of them being characterised by corresponding changes in insulin secretion and insulin sensitivity: compensation, β-cell adaptation, early decompensation, stable decompensation and severe decompensation. The progressive β-cell failure is closely associated with oxidative stress as the expression levels of antioxidant enzymes in β-cells are very low (Robertson, 2004; Kaneto *et al.*, 2005; Prentki & Nolan, 2006). Accordingly, chronically excessive levels of reactive oxygen species are shown to cause decreased insulin gene expression and accelerated rates of β-cell apoptosis (Robertson, 2004). Taking into account the proposed stages of β-cell dysfunction in humans, our results show that two months after castration obese rabbits are in the stage of compensation as they exhibited marked hyperinsulinaemia, higher $HOMA_{\beta\text{-cell}}$, higher insulin secretion rate during the first hour after glucose infusion ($AUC_{insulin\ 0\rightarrow 60min}$).

We found no group differences in the insulin estimates characterising the first or early phase of insulin secretion ($\Delta I/\Delta G$; I_{10min}/G_{10min}; $AUC_{insulin\ 0\rightarrow 10min}$). Therefore, these results suggest that during the early phase of insulin secretion the sensitivity of β-cells to glucose in castrated obese rabbits is probably still preserved but the secretory capacity of β-cells is disturbed, leading to the secretion of inadequate quantity of insulin which is unable to decrease blood glucose to the levels in non-obese rabbits (Georgiev *et al.*, 2011). In chronically obese cats the first phase of insulin secretion was also decreased, while in humans insulin response to exogenous glucose might be normal, increased or reduced (Nelson *et al.*, 1990; Festa *et al.*, 2008). The discrepancy of the obtained results could be at least partly due to the differences in the duration of the obesity period. However, the situation

remains controversial as recent data in humans based on the comparison of surrogate markers (basal and from IVGTT) of insulin secretion and insulin sensitivity show that subjects with impaired glucose tolerance, before the clinical symptoms of diabetes may have more pronounced β-cell defects than previously estimated by homeostasis model assessment (Festa *et al.*, 2008).

The well marked positive correlation between $\Delta I/\Delta G$, I_{10min}/G_{10min} and $AUC_{insulin\ 0\rightarrow10min}$ indicates that each of these indexes could be used for evaluation of first phase of insulin secretion. On the other hand, the strong correlation between fasting insulin and $HOMA_{\beta\text{-cell}}$ indicates that increased insulin concentration at baseline might be considered as a simple and reliable marker not only for insulin resistance but also for β-cell compensation.

In non-castrated non-obese rabbits, lipid content in SMB muscle tended to be higher than in LL muscle. Therefore, more marked increase of lipid content in LL muscle after castration is probably due to its larger capacity for fat deposition.

The castrated rabbits exhibited typical signs of visceral type of obesity (greater body weight, body mass index and amount of intra-abdominal fat) (Georgiev *et al.*, 2011) which is accompanied with marked accumulation of fat in skeletal muscles. In addition, intramuscular lipid content in SMB muscle correlated negatively with glucose elimination rate and positively with visceral fat and weight, indicating that in rabbits, abnormal ectopic fat deposition seems to play a primary pathogenic role in the obesity-induced impairment of insulin sensitivity.

There is increasing body of evidence that reactive oxygen species affect negatively insulin signalling pathway directly (stimulation of serine instead of tyrosine phosphorylation of IR and insulin receptor substrate 1 (IRS-1), increased IRS protein degradation, impaired signal transmission from phosphatidylinositol 3-kinase to protein kinase B, decreased GLUT-4 gene expression) and/or through suppression of adiponectin and peroxisome proliferator-activated receptor gamma and induction of IL-6 and TNF-α gene expressions (Bashan *et al.*, 2009). Nevertheless, a definitive estimation of the impact of dietary antioxidants on obesity-induced disorders such as insulin resistance and β-cell dysfunction is still lacking as the obtained results are inconvincing.

In our study the values of some of surrogate indexes of insulin resistance (basal insulin concentration, I_0/G_0 ratio, $HOMA_{ins.\ resist.}$) in Immunoprotect-treated rabbits were significantly lower while Bennett and QUICKI indexes were significantly higher than in castrated obese rabbits and did not differ from those in non-castrated non-obese rabbits. These results suggest that the supplementation of a combination of two antioxidants (vitamin E and d-limonene) can at least partly restore insulin sensitivity, confirming our previous results (Georgiev *et al.*, 2009; 2011). Our findings correspond to the results of other authors, showing amelioration of oxidative stress-induced insulin resistance by exogenous administration of high doses of vitamin E (Manning *et al.*, 2004; Houstis *et al.*, 2006; Singh *et al.*, 2008) and more recently of d-limonene in rats (Santiago *et al.*, 2011).

The rabbits treated with Immunoprotect exhibited normalisation of β-cell function as the values of $HOMA_{\beta\ cell}$ and $AUC_{insulin\ 0\rightarrow60min}$ were significantly lower than in castrated obese subjects and similar to those in controls. The

favourable effect of Immunoprotect on insulin secretion is probably due to the ameliorated insulin action in treated animals, as there is a close interrelation between β-cell function and insulin sensitivity as shown in human studies (Bergman *et al.*, 2002; Prentki & Nolan, 2006). In addition, a direct beneficial effect of antioxidant treatment on β-cell function could be expected because of the lower local expression of antioxidant enzymes.

The protective effect of Immunoprotect treatment could be in part due to the powerful antioxidant properties of vitamin E and d-limonene, leading to rapid utilisation and degradation of fatty acids (Georgiev *et al.*, 2009), thus inhibiting the lipogenesis and lipid accumulation in muscles. This is confirmed by our results from chemical muscle lipid analysis showing a marked decrease in response to Immunoprotect. Recently, d-limonene supplementation in high-fat diet-induced obesity in rats has been shown to decrease hepatic fat deposition throughout reduction of the activities of the key enzymes involved in the synthesis of fatty acids and triglycerides (Santiago *et al.*, 2011).

In conclusion, we demonstrated that basal insulin concentration, $HOMA_{ins\ res}$, I_0/G_0 ratio, $HOMA_{\beta}$ cell, $AUC_{insulin\ 0\rightarrow60min}$ and intramuscular lipid content in castrated obese rabbits were significantly higher while QUICKI and Bennett indices were significantly lower than in non-castrated non-obese rabbits. Surrogate indices are simple and reliable indicators of insulin sensitivity and β-cell function in rabbits as they were closely associated with markers of obesity and can be modified by antioxidant supplementation.

ACKNOWLEDGEMENTS

This study was supported by grant from the Ministry of Education and Science of Bulgaria and Trakia University Science Foundation, Stara Zagora, Bulgaria (grant no. 10/06). We would like to greatly acknowledge Prof. Zahari Raikov MD from the Medical Faculty of the Trakia University, Stara Zagora, Bulgaria for providing Immunoprotect and for his great and valuable scientific support, confidence and for the successful collaboration.

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Paper received 11.12.2014; accepted for publication 22.01.2015

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