HYPERBARIC OXYGEN AND INSULIN SENSITIVITY

David Cameron Wilkinson

Adelaide Medical School
The University of Adelaide

September, 2020

THESIS DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

I acknowledge that copyright of published works contained within this thesis resides with the copyright holder(s) of those works.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

David Cameron Wilkinson

4/9/20

ACKNOWLEDGEMENTS

I would like to thank the many people who have helped me over the years. First, my supervisors: Leonie Heilbronn, Ian Chapman and Guy Ludbrook. Leonie Heilbronn and Ian Chapman have also been my co-investigators and I thank them for their patience and guidance. My special thanks go to Leonie's laboratory team whose assistance has been invaluable.

Thanks to John Lippmann for his support in general and his assistance in constructing the final document.

Thanks to the Nurses and Technicians of the Hyperbaric Medicine Unit of the Royal Adelaide Hospital who always helped when asked.

And finally,
this thesis is dedicated to
my wife,
Mandy.

TABLE OF CONTENTS

			Page
Title page	……		i
Declaration	……		iii
Acknowledgement	……		iv
Contents	……		v
Published papers	……		vii
Abstract	……		viii
Introduction	……		1
Chapter 1	Diabetes, insulin resistance, inflammation and hyperbaric oxygen - a literature review (unpublished)		3
Chapter 2	a.	Authorship statement	23
	b.	Publication #1: Hyperbaric oxygen therapy improves peripheral insulin sensitivity in humans	25
Chapter 3	a.	Introduction	29
	b.	Authorship statement	30
	c.	Publication #2: Hyperbaric oxygen therapy increases insulin sensitivity in overweight men with and without type 2 diabetes	32
Chapter 4	a.	Introduction	39
	b.	Authorship statement	41
	c.	Publication #3: Assessment of insulin sensitivity during hyperbaric oxygen therapy	43

Chapter 5
a. Introduction 56
b. Authorship statement 57
c. Inflammatory cytokine production and adipose tissue gene expression in response to hyperbaric oxygen – unpublished data 59

Chapter 6
a. Introduction 74
b. Authorship statement 75
c. Publication #4: Hyperbaric oxygen but not hyperbaric air increases insulin sensitivity in men with type 2 diabetes mellitus 76

Conclusions and recommendations ……. 88

PUBLISHED PAPERS

The candidate was the principal author of the following publications in peer-reviewed journals which are submitted as part of this thesis:

Wilkinson D, Chapman IM, Heilbronn LK. Hyperbaric oxygen therapy improves peripheral insulin sensitivity in humans. Diabet Med. 2012;29(8):986-9. doi: 10.1111/j.1464-5491.2012.03587.x. PubMed PMID: 22269009.

Wilkinson D, Nolting M, Mahadi MK, Chapman I, Heilbronn L. Hyperbaric oxygen therapy increases insulin sensitivity in overweight men with and without type 2 diabetes. Diving Hyperb Med. 2015;45(1):30-6. PubMed PMID: 25964036.

Wilkinson D, Szekely S, Gue B, Tam CS, Chapman I, Heilbronn LK. Assessment of Insulin Sensitivity during Hyperbaric Oxygen Therapy. Diving Hyperb Med. (accepted for publication 18 March 2020).

Wilkinson DC, Chapman IM, Heilbronn LK. Hyperbaric oxygen but not hyperbaric air increases insulin sensitivity in men with type 2 diabetes mellitus. Diving Hyperb Med. (accepted for publication 22 August 2020)

ABSTRACT

Introduction

Obesity is associated with a chronic low grade inflammatory state and the development of insulin resistance and diabetes, while hyperbaric oxygen therapy (HBOT) has demonstrated anti-inflammatory properties. But it was the observation that people with diabetes were susceptible to a fall in blood glucose levels during HBOT that led to an investigation of insulin sensitivity.

Methods

Five human studies were reported in four publications. Four studies used the hyperinsulinaemic euglycaemic glucose clamp and one used a frequently sampled intravenous glucose tolerance test (FSIGT) to investigate insulin sensitivity during HBOT. Studies recruited men who were overweight or obese, with and without diabetes. Blood samples for inflammatory cytokines and adipose tissue biopsies for gene expression were taken to investigate possible mechanisms of action.

Results

A total of sixty-two men were investigated by glucose clamp and nine by FSIGT. All four glucose clamp studies showed significant within-group increases in insulin sensitivity following exposure to HBOT. First, in a group of patients with (n=5) and without diabetes (n=5) referred for clinical HBOT, there was a 37% increase during the third HBOT and 41% increase during the thirtieth HBOT. Next, in a group of men who were overweight or obese, we found a 29% increase in men without diabetes (n=11) and 57% increase in those with diabetes (n=8) during the third HBOT. Further, the effect was still measurable in the first 30 minutes after exit from the hyperbaric chamber. Pre and post-HBOT testing found reductions in serum TNF-α and MCP-1 while IL-6 increased. Next, in a group of men who were overweight or obese but without diabetes (n=9), we found a significant 23% increase during the first HBOT intervention. There was a significant increase in serum IL-6 after HBOT but no change in TNF-α or MCP-1. The final glucose clamp study randomised men to either HBOT (n=13) or hyperbaric air (n=11) and found a significant between group difference with a 26% increase in insulin sensitivity during HBOT but no significant change in hyperbaric air. The FSIGT study performed the insulin sensitivity test on men who were

overweight or obese and without diabetes (*n*=9) during the third HBOT and 24-hours later but found no changes from baseline.

Conclusions

The glucose clamp technique identified an acute increase in insulin sensitivity during HBOT. The effect could be seen in those without diabetes which suggests it is a physiological response to HBOT. Further research is encouraged into this insulin-sensitising effect of HBOT as it could open new therapeutic pathways for glucose regulation. No change to insulin sensitivity was seen in hyperbaric air; although it was a very modest exposure this may be relevant to people in other hyperbaric environments such as diving. The response of serum inflammatory cytokines to HBOT was inconsistent however two studies that showed increased insulin sensitivity during HBOT together with increased serum IL-6 suggest the origin and role of IL-6 requires further investigation.

INTRODUCTION

The reader may be assisted by a few words to explain the layout of this thesis and the reasoning that created it. When this journey began, the observation that people with diabetes were at risk of significant falls in their blood glucose level during clinical use of Hyperbaric Oxygen Therapy (HBOT) had been made in several publications. All hyperbaric facilities had protocols in place to monitor the blood glucose level of anyone with diabetes before they entered the chamber and food was provided to those with low or borderline readings prior to commencing HBOT. However, there appeared to have been no serious attempt to investigate why this occurred. I was attracted to this question.

I initially discussed strategies to investigate this as a pilot study in patients referred for HBOT. I sought advice from an Endocrinologist; he helped me with the technical requirements of a hyperinsulinaemic euglycaemic glucose clamp and later became my principal supervisor. It became apparent during this pilot study that HBOT was having an influence on the glucose infusion rate during the glucose clamp technique and a more comprehensive study design was required. I felt that if I was to undertake further clinical studies in this topic, I should structure this investigation as a formal program towards a higher degree by research with the University of Adelaide.

The pilot study was completed and published early in my candidature (Chapter 2). The literature review (Chapter 1) was written on the completion of this study and was influenced by the study findings at that point in time. The literature review was not written with a view to publication. With specialist medical training in Anaesthesia and Hyperbaric Medicine, the literature review was an opportunity for me to understand diabetes and the intracellular glucose transport pathway in much greater detail than I ever had before. The literature review also provided a section on HBOT to orientate the reader who was not familiar with this field of medicine, as well as a discussion about the actions of HBOT, particularly in regard to evidence for an anti-inflammatory action. This intersected with the considerable evidence for inflammatory cytokines released from adipose tissue driving a chronic low grade inflammatory state that has been linked to the development of insulin resistance and type 2 diabetes. As this program of research

developed, the association of an anti-inflammatory action of HBOT with an insulin-sensitising effect of HBOT has become less clear.

I have not revised the literature review in the intervening years. I regard the literature review (Chapter 1) as a primer for the focus of our thinking at the time and I have followed this directly with the published first study (Chapter 2). Thereafter, I have inserted a short introduction before each publication to provide some contextual discussion about our thinking at the time and justification for the subsequent study design.

CHAPTER 1

Diabetes, insulin resistance, inflammation and hyperbaric oxygen

A literature review

Diabetes is a significant and increasing burden not just on the health system but ultimately the entire society for both developed and developing nations. In 2012 it was estimated that there were 22.3 million people in the US with the diagnosis of diabetes, approximately 7% of the population[1]. This has increased from an estimated 17.5 million in 2007. In 2012, the cost of health care for diabetes and associated lost productivity was estimated at US$245 billion, an increase of 41% from 2007. In Australia there are approximately 1.8 million people with diabetes and by 2031 it is estimated that 3.3 million Australians will have type 2 diabetes mellitus (T2DM)[2]. Government health budgets around Australia spend Aus$6 billion a year on diabetes without considering personal costs or lost productivity. On a global perspective, health expenditure on diabetes was estimated at US$376 billion in 2010 and almost 4 million deaths in the 20-79 age group were attributed to diabetes-related causes.

Type 1 diabetes mellitus (T1DM) and T2DM are the two major forms of this disease. T1DM is an auto-immune disease with destruction of the β-cells of the pancreatic islets resulting in reduced or absent insulin secretion. It tends to occur in childhood or adolescence and accounts for about 10% of people with diabetes. T2DM accounts for approximately 90% of cases and is characterised by reduced tissue responsiveness to the actions of insulin together with β-cell dysfunction which reduces the capacity of the pancreas to secrete sufficient insulin. There is a multifactorial genetic component to T2DM however it is also strongly influenced by the environment, particularly diet and lifestyle factors.

The relative impairment of the action of insulin on target tissues, notably muscle and liver, defines insulin resistance. The inverse of insulin resistance is termed insulin sensitivity. The onset of insulin resistance in target tissues elicits a compensatory response from the pancreas with an increase in insulin secretion. Insulin secretion can increase four to five-fold and β-cell mass can increase by 50% to augment the stimulus required to maintain normal blood glucose levels[3]. Insulin sensitivity can vary throughout life and insulin resistance has been observed physiologically during puberty, pregnancy

and with normal ageing. Increased insulin sensitivity can be seen with increased physical activity.

For insulin resistance to progress to diabetes, β-cell dysfunction is required. A combination of genetic and environmental factors can render individuals susceptible to β-cell changes which result in a diminished capacity to secrete insulin. Progressively, euglycaemia will be replaced by impaired glucose tolerance. With further reduction in insulin secretion, hyperglycaemia and clinical diabetes can eventually occur. Not everyone with insulin resistance will develop diabetes however insulin resistance is the best predictor of those likely to develop diabetes in the future [4].

The epidemic in diabetes is paralleled by an epidemic in obesity. The US-based Centers for Disease Control and Prevention (CDC) estimated that the proportion of the US population that are obese (BMI≥30kg/m2) was 18% in 1998, 25% in 2006 and 35% in 2009. In 2009, 17% of youth were considered obese. The financial burden of obesity consumed almost 10% of all medical spending in 2008, around US$147 billion[5]. In Australia, adult prevalence of those overweight (25≤BMI<30) and obese has increased from 56% in 1995, to 61% in 2007 and just over 63% in 2011 (ABS). For 2008, the total costs of obesity including medical costs, lost productivity and carers cost was estimated at Aus$58 billion.

Chronic inflammation

Obesity has a strong association with both insulin resistance and the development of T2DM. Obesity-related insulin resistance is associated with a chronic inflammatory state [6]. Physiologically, we understand inflammation to be a short-term adaptive response to injury and infection by both the metabolic and immune systems. Obesity produces a sustained metabolic and immune response which shares many of the same chemical mediators as classical inflammation. The consequences of this prolonged inflammatory state may lead to the development of a cluster of metabolic diseases, or so-called metabolic syndrome – insulin resistance, T2DM, dyslipidaemias, hypertension and accelerated cardiovascular disease. The CDC estimates that 20% of the adult population in the US has the metabolic syndrome.

There is an abundance of evidence linking obesity with chronic inflammation and insulin resistance. The first link was published in 1993 when the inflammatory cytokine, tumour

necrosis factor(TNF)-α was shown to be overexpressed in the adipose tissue of obese mice [7]. When a TNF-α receptor blocker was given to these mice the insulin resistance was reversed, requiring 2-3 times the glucose infusion rate during hyperinsulinaemic euglycaemic clamp studies. This resistance predominantly affected peripheral glucose utilisation as opposed to hepatic glucose output. Another study showed that knockout TNF-α mice do not develop insulin resistance despite obesity[8]. Overexpression of TNF-α has been identified in the adipose tissue of obese humans and weight loss led to a reduction in TNF-α[9, 10]. Overexpression of TNF-α has also been identified in the muscle of obese humans[11]. Infusion of TNF-α induces insulin resistance in humans[12] specifically at the level of the skeletal muscle[13].

Adipose tissue comprises adipocytes plus a stromal tissue portion including connective tissue, preadipocytes, blood vessels and macrophages. Adipose tissue from obese animals and humans differs from that in lean subjects with infiltration by macrophages. It is estimated that fat tissue of lean humans contains about 10% macrophages but in obese humans it can contain up to 40% macrophages[14]. Almost all of the over-expressed TNF-α in obesity can be identified as coming from the macrophage population within adipose tissue[15, 16].

So a picture of obesity emerges with an increase in fat tissue mass being associated with macrophage infiltration and activation leading to up regulation of TNF-α production and a sustained inflammatory response[17]. Other local effects may include TNF-α triggering the conversion of preadipocytes into macrophages with the expression of further inflammatory cytokines[15]. Cytokines released into the systemic circulation can then lead to the development of insulin resistance particularly at the level of skeletal muscle. Furthermore, it has also become apparent that TNF-α is not the only inflammatory cytokine released in obesity but includes monocyte chemmoattractant protein-1 (MCP-1), and several members of the interleukin (IL) family. On the flip side, anti-inflammatory cytokine production including adiponectin may be reduced in obese individuals and promote insulin resistance.

The pro-inflammatory cytokine, MCP-1 is secreted by a range of cells including adipocytes, macrophages and vascular endothelial cells. It is a potent attractant for monocytes and macrophages and MCP-1 expression in adipose tissue is increased in the setting of obesity[14, 15]. Mice rendered obese by a high-fat diet and transgenic MCP-1 mice

demonstrate macrophage infiltration of fat tissue and systemic insulin resistance by clamp studies[18, 19]. In another study using transgenic MCP1 mice, insulin resistance was associated with increased expression of TNF-α and Il-6 in adipose tissue[20]. Chronic administration of MCP-1 led to insulin resistance together with macrophage infiltration in mice while acute MCP-1 administration caused insulin resistance without macrophage infiltration. This suggests that MCP-1 may have a direct effect on insulin signalling that is independent of macrophage activity[21].

IL-6 has traditionally been considered a pro-inflammatory cytokine however emerging evidence suggests its role is far more complicated. IL6 is produced by many diverse cell types although one third of basal IL-6 secretion comes from macrophages within adipose tissue[6, 14, 22] and mostly from visceral rather than subcutaneous adipose tissue[16, 23]. IL-6 levels are increased with normal ageing in the absence of disease; it is responsive to sex hormones with actions on the menstrual cycle and spermatogenesis[24] and it has CNS interactions in relation to psychological stress, fatigue and sleep[25]. Even in the context of insulin response it appears to display both pro- and anti-inflammatory effects and is now considered a pleiotropic cytokine. The pro-inflammatory role for IL-6 is supported by obese women who have increased serum levels of IL-6 also show reduced insulin sensitivity as measured by the fasting insulin resistance index[26]. The degree of insulin resistance was better predicted by the IL-6 level than by other cytokines such as TNF-α. Weight loss led to a reduction in IL-6 level together with an improvement in the insulin response without a significant change in TNF-α. In another study, human obesity was associated with increased IL-6 in adipose tissue and serum however it was serum IL-6 that had the closest relationship with reduced insulin sensitivity as measured by intravenous glucose tolerance test[27]. A more complex role for IL-6 is suggested by the finding that exercise is an insulin sensitiser[28] and is associated with the release of IL-6 from muscle where serum concentrations can increase by up to 100-fold[29]. Also, acute infusion of IL-6 in humans led to increased insulin sensitivity measured by hyperinsulinaemic euglycaemic clamp[30]. In another clamp study, infusion of IL-6 showed no change in insulin sensitivity together with no increased gene expression of IL-18, another pro-inflammatory cytokine linked to insulin resistance[12]. A further study has suggested IL-6 may have differing effects in different tissues, showing that while IL-6 improved glucose uptake in muscle, it negatively influenced vascular endothelial cells[31].

IL-1 is a pro-inflammatory cytokine with two subtypes; IL-1α and IL-1β. They have similar but not identical actions however both have been shown to interfere with insulin signalling using in vitro models[32-34]. IL-1 is produced at sites of inflammation together with TNF-α and they act synergistically[35]. IL-1β has been demonstrated to be particularly detrimental to pancreatic islet β-cells[36]. IL-1 receptor antagonist (IL-1ra) is a member of the IL-1 family, produced mainly by adipocytes and binds to the IL-1 receptor antagonising its action[37]. There is a balance between IL-1 and IL-1ra activity. Levels of IL-1ra are increased in human obesity, reduced by weight loss and are related to insulin resistance as measured by the insulin resistance index[38]. Use of analogues of IL-1ra in people with T2DM has resulted in lower glycosylated haemoglobin levels, improved β-cell secretory function but no change in insulin sensitivity by clamp[39].

IL-18 is a pro-inflammatory cytokine and member of the IL-1 family. IL-18 plasma levels are elevated in people with T2DM compared to those without diabetes and IL-18 levels correlate with homeostasis model assessment of insulin resistance (HOMA-IR)[40]. Another study found plasma IL-18 is increased in obesity, reduced with weight loss and was correlated with HOMA -IR[41]. In a population study of 955 people, serum IL-18 levels not only correlated with BMI and waist circumference but also with metabolic syndrome[42].

This is not an exhaustive list of players however this soup of cytokines already suggests a more complicated, inter-related process is in play. The weight of evidence for TNF-α having a major role is evident although human studies using drugs to block TNF-α action have not been so conclusive[43, 44]. IL-6 can have pro- and anti-inflammatory actions yet some of the studies mentioned show IL-6 levels are a better predictor for insulin resistance than other cytokines like TNF-α. Certainly there are differences in the effects of short-term infusions of IL-6 compared to chronic infusions. Cytokines are known to work in networks - the effect of any particular chemical is dependent on its position in the hierarchy of the network and combinatorial and additive relationships are crucial[45]. Another author suggested that "the final effect of ILs on insulin sensitivity is likely to depend on a subtle balance of their relative concentrations (high or low), kinetics (acute or chronic) and targets (central nervous system, liver, adipose tissue, skeletal muscle and pancreas)"[46].

Cytokines directly inhibit the insulin signalling cascade

The process whereby insulin promotes glucose uptake is as follows; the insulin receptor floats in the muscle cell membrane, a 340,000D molecular weight tetramer encoded by a single gene. The two α-subunits sit outside the cell membrane and are attached to the two β-subunits by disulphide bonds. The two β-subunits span the membrane and within the cell display tyrosine kinase activity. When insulin binds to the α-subunits the tyrosine kinase undergoes autophosphorylation which then allows interaction with other intracellular proteins, including insulin receptor substrate(IRS)-1 and IRS-2. The activated substrates recruit additional kinases and phosphatases in a complex signalling pathway that includes phosphatidylinositol-3-kinase (PI3K). PI3K activates the serine/threonine kinase Akt (Protein Kinase B) which induces the translocation of vesicles containing the glucose transporter GLUT4 to the cell membrane. The GLUT4 receptors rapidly appear on the cell membrane within minutes of insulin stimulation and come from supplies of newly formed as well as recirculated receptors stored in vesicles. Signalling is switched off at multiple levels including dissociation of insulin and internalisation of the receptor; the action of intracellular protein tyrosine phosphatases such as PTP1b which deactivates the tyrosine kinase on the insulin receptor; the blocking of further activation of IRS-1 and other sites downstream; and Akt itself which can inhibit upstream insulin signalling. The mechanism of insulin resistance may act at several of these levels also.

Cytokines, including TNF-α, IL-6 and IL-1 bind to membrane-bound receptors activating several intracellular protein kinases, notably c-Jun amino-terminal kinase (JNK) and inhibitor of nuclear factor κB kinase (IKK)[3, 6, 45]. Both lead to serine phosphorylation of IRS-1, which then prevents IRS-1 undergoing tyrosine phosphorylation on the insulin receptor and activating the insulin-signalling pathway. JNK and IKK interfere with signalling at other levels of the pathway and also influence gene expression leading to further cytokine production via effects on activator protein-1 and NFκB. Cytokines may induce other pathways that produce insulin resistance such as suppressor of cytokine signalling (SOCS) proteins and inducible nitric oxide synthase (iNOS).

The chronic inflammatory process associated with obesity is also associated with increased lipid accumulation in muscle, liver and blood. While not the focus of this review, lipids may activate protein kinase C, which can lead to serine phosphorylation of IRS-1 as well as activate other kinases[47]. Lipid interaction with fatty-acid-binding proteins and nuclear receptors can also induce further inflammatory cytokine expression.

However even the role of lipids is complicated by lipid-ligands to transcription factors such as the PPAR and liver X receptor families which can suppress inflammatory cytokine expression[6, 45].

The trigger for inflammation

The chronic inflammatory response seen with obesity appears to originate in, and is largely driven by, the increasing adipose tissue mass[6, 45]. The reason why an increase in adipose tissue should trigger such an inflammatory response is not clear. Theories have suggested factors such as hypoxia, oxidative stress and endoplasmic reticulum (ER) stress, although there is clearly some overlap between these.

It is proposed that obesity leads to hypoxia of adipose tissue due to a combination of increasing adipose cell size, reduced capillary density and limited diffusion distance of oxygen[48]. The cellular response to hypoxia includes activation of hypoxia inducible factor(HIF)1-α leading to the coordinated expression of a number of adaptive genes, including inflammatory mediators. In support of this, the adipose tissue of obese mice was found to have reduced blood flow and lower oxygen tension than that in lean mice, and over-expression of a number of stress responsive genes[49]. Further, polarographic Clark electrodes inserted into obese human adipose tissue found lower oxygen levels and increased insulin resistance as measured by hyperinsulinaemic euglycaemic clamp[50]. However the change in adipose tissue oxygen did not correlate with the glucose dispersal rate. Obstructive sleep apnoea is characterised by repeated episodes of hypoxia and has been associated with increased levels of TNF-α, CRP and IL-6 independent of obesity[51, 52]. Treatment with nasal CPAP reduced the levels of these inflammatory mediators. In another study, non-obese male volunteers were found to develop insulin resistance measured by clamp after 2 days of exposure to the relative hypoxia of high altitude however this was largely compensated by 7 days[53]. Non-obese male volunteers were also found to develop insulin resistance by clamp studies after a 30-minute exposure to hypoxia sufficient to lower SpO2 from 96% to 75%[54]. While significant hypoxia appears to induce insulin resistance, it is unclear whether the lower oxygen level recorded in obese adipose tissue is actually a metabolic stress. Hypoxic stress may be indicated by an increase in lactate effusion from the adipose tissue suggestive of increased glycolysis and decreased oxidation of glucose for metabolism. A recent study using tissue-specific

venous catheterisation found that obese adipose tissue did have reduced oxygen delivery but has low oxygen demand and showed no metabolic evidence of hypoxia[55].

Oxidative stress may be precipitated by the increased nutrient supply to adipose tissue. The increasing amount of glucose transported across the endothelial cell membranes places a large metabolic strain on the mitochondria which results in increased production of reactive oxygen species (ROS)[6]. The rise in intracellular ROS activates inflammatory signalling cascades which lead to macrophage attraction and infiltration, exacerbating the inflammatory response.

The endoplasmic reticulum (ER) is an organelle that serves multiple functions in the cell. Proteins are synthesized on the cytoplasmic surface of the ER, and those destined for secretion or for transmembrane compartments are translocated into the ER, where they undergo posttranslational modifications such as disulphide bond formation or glycosylation, and are correctly folded and assembled to their final three dimensional conformation. Factors that interfere with ER function, such as lipids, increased synthesis of secretory proteins, infection or hypoxia lead to accumulation of unfolded or misfolded proteins. ER stress triggers the unfolded protein response (UPR) with the goal of rescuing the cell. The UPR is typically regulated by three transmembrane proteins, PERK (PKR-like ER-resident kinase), ATF6 (activating transcription factor 6) and IRE1 (inositol-requiring enzyme 1). The activation of these enzymes leads to a range of changes in cell function but includes increased ROS generation, activation of JNK and IKK pathways and increased nuclear transcription factors stimulating further inflammatory cytokine production[45, 56].

So far, the discussion linking a chronic inflammatory state with insulin resistance has been in association with obesity. It is pertinent to note that not all people with diabetes, or with insulin resistance, are obese. One study has looked at the relationship between inflammatory markers and insulin sensitivity in a large population cohort (1,221 males aged about 70 years)[57]. Insulin sensitivity was measured by hyperinsulinaemic euglycaemic clamp. The whole cohort did demonstrate that higher serum levels of CRP and IL-6 were associated with reduced insulin sensitivity. Amongst a cohort of men with normal BMI and no diabetes or metabolic syndrome (n=382) the relationship of raised CRP and IL-6 with reduced insulin sensitivity was still maintained. This indicates that a

state of chronic inflammation is independently associated with insulin sensitivity; it does not necessarily have to be in association with obesity.

Hyperbaric oxygen treatment

Hyperbaric oxygen treatment (HBOT) is the breathing of 100% oxygen while exposed to increased atmospheric pressure. It involves placing a person in a steel chamber which is pressurised usually to between 2 to 3 atmospheres absolute (ATA), with a hood or mask system providing 100% inspired oxygen. Treatments last 90-120 minutes and are typically provided as a course of 20-40 sessions. HBO has been a recognised therapy in the management of decompression illness and air embolism. Increasingly, clinical studies are demonstrating a benefit for a variety of medical conditions including non-healing wounds (particularly in people with diabetes)[58], tissue damage as a result of radiotherapy[59] and necrotising tissue infections[60]. The hyperoxia will provide vital oxygen to tissue with marginal perfusion however there is increasing interest in HBOT's ability to influence cell signal transduction cascades[61]. The supraphysiological levels of oxygen achieved with HBOT have been shown to stimulate secretion of growth factors, VGEF and heat shock proteins, mobilise stem cells and modulate specific activity of neutrophils.

A proportion of patients referred for HBOT have diabetes and it has been an observation that people with diabetes tend to experience a fall in their blood sugar level (BSL) during HBOT exposure. Six patients with diabetes had their BSL followed by laboratory glucometer over the course of a 2-hour HBOT[62]. BSL fell by a mean 3.5 mmol/l with no change in serum insulin. Another study compared 237 glucometer BSLs pre and post-HBOT in patients with diabetes, finding a mean fall of 2.04mmol/l[63]. The change in BSL was greater in those taking insulin compared to oral hypoglycaemics, and there was no change in HbA1c. Another study followed 41 patients with diabetes and found a mean fall in BSL of 23%[64]. The mechanism of this effect has not been examined and it is not apparent whether this is specific to those with diabetes or whether those without diabetes are able to compensate for it. Since a chronic inflammatory state is strongly associated with reduced insulin sensitivity, it is appropriate to consider what is known about the effect of HBOT on immune function.

Most studies of HBOT demonstrate suppression of inflammation although a few suggest an enhanced response. One study using healthy subjects and a brief compression to 2 and

2.8ATA breathing air or oxygen found a doubling of TNF-α while at pressure but which was not evident 30-minutes later[65]. There was evidence of lipid peroxidation but no change in serum IL-6 and IL-1β. Two other studies using an in vitro macrophage preparation found that prior HBOT exposure induced an exaggerated release of TNF-α when the macrophages were stimulated by lipopolysaccharide (LPS) exposure[66, 67].

HBOT has demonstrated immunomodulation in many studies using several different models of inflammatory injury. In a mouse study using caecal ligation and puncture as a model for septic peritonitis, 12-hourly HBOT was associated with improved survival possibly by mechanisms involving increased IL-10[68]. Survival was maximal at a pressure of 2.5ATA with no survival advantage at lower pressures and increased mortality at higher pressures, suggesting that the protection is dose dependent. In a mouse model bred for the development of atherosclerosis, daily HBOT at 2.4ATA for 5 weeks reduced the degree of histological change in the wall of the aorta, while also reducing inflammatory markers and increasing antioxidant pathways[69]. Spleen cells from the HBOT group showed increased levels of IL-10 while LPS activation led to reduced expression of TNF-α, IL-6 and IL-1β. A further rat study used zymosan injected into the peritoneum to induce a model of multiple organ dysfunction[70]. Two sessions of HBOT at 2ATA reduced the degree of visible peritonitis as well as reduced plasma levels of TNF-α and IL-1β. There was reduced expression of TLR-2 and TLR-4, a pathway known to activate NFκB. An in vitro human monocyte model exposed to HBOT at 2.4ATA and stimulated with LPS revealed reduced expression of TNF-α and IL-1β[71]. Interestingly, this effect was only found for HBOT at 2.4ATA, there was no change in monocytes exposed to normobaric oxygen or to pressure alone. Also, the degree of suppression in the HBOT group was related to the duration of exposure. Suppression was maximal after 30-minutes, continued exposure for 3-hours returned the response to baseline while exposure for 12-hours produced even greater release of inflammatory markers. While these studies used different animal species and different models, they point to the importance of the oxygen "dose" determined by the pressure and duration of HBOT exposure. Normobaric oxygen exposure (ie 100% oxygen at 1ATA) does not induce immunomodulation while exposure to oxygen levels that are too high or for too long can worsen the outcome, presumably related to the onset of oxygen toxicity. Within this therapeutic window HBOT may suppress certain immune system responses to inflammatory stimuli. However, it is important to appreciate that very specific cell functions are affected

without a global effect on the immune system as a whole that could lead to immunocompromise. For example, HBOT has been shown to reversibly interfere with CD11/18 activity on the neutrophil membrane, an effect which has been utilised in the clinical management of ischaemia-reperfusion injury[72]. However the neutrophil will still respond normally to endotoxin and LPS exposure and phagocytosis and related activities are preserved.

Reactive oxygen species

Adipose tissue that is subjected to stress, either oxidative or by the ER increasing ROS production, activates other pro-inflammatory pathways and reduces insulin sensitivity. ROS have generally been thought of as the toxic waste product of oxidative cell metabolism and only likely to induce a negative change, such as reduced insulin sensitivity. It seems counterintuitive to then suggest that HBOT may be beneficial when HBOT undoubtedly generates a large amount of ROS and reactive nitrogen species (RNS)[61]. Important in reconciling this conflict is the realisation that ROS and RNS are also signalling molecules in the transduction cascades and pathways of cell activity. HBOT exposures are relatively brief while at the same time HBOT acutely enhances the antioxidant responses in tissue with effects on superoxide dismutase and catalase. There appears to be a subtle interplay between the oxygen dose and its tissue-specific effect on the opposing influences of increased ROS and enhanced anti-oxidant activity. A degree of oxidative stress seems crucial to the action of HBOT however oxygen toxicity could negate the benefit.

The concept that some oxidative stress may be beneficial is reflected in the experimental use of HBOT as a preconditioning agent. Preconditioning is the prophylactic use of a manoeuvre to better prepare tissue for a known, deliberate challenge. It is most commonly employed in the setting of cardiac bypass surgery or organ transplantation where tissues will suffer a period of ischaemia. Preconditioning manoeuvres have included a brief period of surgically-induced ischaemia and use of chemical agents such as carbon monoxide. HBOT used prior to cardiac surgery has resulted in improved cardiac function post-operatively with evidence of a reduction in post-operative inflammation[73, 74].

A recent article has further complicated the ROS story by suggesting that ROS have the capacity to both promote and attenuate the insulin response[75]. There is clear evidence for

the association of obesity and T2DM with oxidative stress, and for ROS being involved in insulin resistance. Essentially, mitochondrial dysfunction and ER stress can lead to intracellular ROS generation with subsequent activation of JNK and IKK pathways. What has now become apparent is that ROS are involved in normal insulin signalling. Insulin, while activating the tyrosine kinase within the insulin receptor, also co-ordinately inactivates the enzymes responsible for turning off the cascade that leads to GLUT4 translocation. These enzymes include members of the protein tyrosine phosphatase family such as PTP1B (which dephosphorylates the insulin receptor tyrosine kinase activation loop) and PTEN (which interrupts PI3K signalling downstream). These enzymes are very susceptible to reversible oxidation. Insulin appears to stimulate the activity of NADPH oxidase within the cell membrane adjacent to the insulin receptor leading to generation of ROS which act locally to inhibit protein tyrosine phosphatase activity and augment the insulin signal. ROS appear to be vital in normal cell signalling as well as propagating pathological processes. Which of these aspects predominate may depend on where in the cell the ROS are generated and whether the response is acute or chronic.

Postscript

At this stage of research, we have one published paper[76] while another is in preparation. In these studies, we have been able to show that 3 sessions of HBOT leads to a significant increase in insulin sensitivity in those with T2DM and further, that it occurs in those without diabetes also. These results suggest our line of investigation has valid scientific merit.

References

1. American Diabetes Association 2013 [cited 2013]. Available from: https://www.diabetes.org/diabetes.
2. Diabetes Australia 2013 [cited 2013]. Available from: https://www.diabetesaustralia.com.au/diabetes-in-australia.
3. Kahn SE, Hull RL, Utzschneider KM. Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature 2006;444:840-6

4. Warram JH, Martin BC, Krolewski AS, Soeldner JS, Kahn CR. Slow glucose removal rate and hyperinsulinemia precede the development of type II diabetes in the offspring of diabetic parents. Ann Intern Med 1990;113:909-15
5. Finkelstein EA, Trogdon JG, Cohen JW, Dietz W. Annual medical spending attributable to obesity: Payer-and service-specific estimates. Health Aff (Millwood) 2009;28:w822-w31
6. Wellen KE, Hotamisligil GS. Inflammation, stress, and diabetes. J Clin Invest 2005;115:1111-9
7. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: Direct role in obesity-linked insulin resistance. Science 1993;259:87-91
8. Uysal KT, Wiesbrock SM, Marino MW, Hotamisligil GS. Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. Nature;389:610-4
9. Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM. Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. J Clin Invest 1995;95:2409-15
10. Kern PA, Saghizadeh M, Ong JM, Bosch RJ, Deem R, Simsolo RB. The expression of tumor necrosis factor in human adipose tissue. Regulation by obesity, weight loss, and relationship to lipoprotein lipase. J Clin Invest 1995;95:2111-9
11. Saghizadeh M, Ong JM, Garvey WT, Henry RR, Kern PA. The expression of TNFalpha by human muscle: Relationship to insulin resistance. J Clin Invest 1996;97:1111-6
12. Krogh-Madsen R, Plomgaard P, Moller K, Mittendorfer B, Pedersen BK. Influence of TNF-alpha and IL-6 infusions on insulin sensitivity and expression of IL-18 in humans. American Journal of Physiology - Endocrinology and Metabolism 2006;291:E108-E14
13. Plomgaard P, Bouzakri K, Krogh-Madsen R, Mittendorfer B, Zierath JR, Pedersen BK. Tumor necrosis factor-alpha induces skeletal muscle insulin resistance in healthy human subjects via inhibition of Akt substrate 160 phosphorylation. Diabetes 2005;54:2939-45

14. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr. Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest 2003;112:1796-808
15. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, et al. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. J Clin Invest 2003;112:1821-30
16. Fain JN, Madan AK, Hiler ML, Cheema P, Bahouth SW. Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. Endocrinology 2004;145:2273-82
17. Heilbronn LK, Campbell LV. Adipose tissue macrophages, low grade inflammation and insulin resistance in human obesity. Curr Pharm Des 2008;14:1225-30
18. Sartipy P, Loskutoff DJ. Monocyte chemoattractant protein 1 in obesity and insulin resistance. Proc Natl Acad Sci U S A 2003;100:7265-70
19. Kanda H, Tateya S, Tamori Y, Kotani K, Hiasa K, Kitazawa R, et al. MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. J Clin Invest 2006;116:1494-505
20. Kamei N, Tobe K, Suzuki R, Ohsugi M, Watanabe T, Kubota N, et al. Overexpression of monocyte chemoattractant protein-1 in adipose tissues causes macrophage recruitment and insulin resistance. J Biol Chem 2006;281:26602-14
21. Tateya S, Tamori Y, Kawaguchi T, Kanda H, Kasuga M. An increase in the circulating concentration of monocyte chemoattractant protein-1 elicits systemic insulin resistance irrespective of adipose tissue inflammation in mice. Endocrinology 2010;151:971-9
22. White UA, Stephens JM. The gp130 receptor cytokine family: regulators of adipocyte development and function. Curr Pharm Des 2011;17:340-6
23. Fried SK, Bunkin DA, Greenberg AS. Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: Depot difference and regulation by glucocorticoid. J Clin Endocrinol Metab 1998;83:847-50
24. Ershler WB, Keller ET. Age-associated increased interleukin-6 gene expression, late-life diseases, and frailty. Annu Rev Med 2000;51:245-70
25. Rohleder N, Aringer M, Boentert M. Role of interleukin-6 in stress, sleep, and fatigue. Ann N Y Acad Sci 2012;1261:88-96

26. Bastard JP, Jardel C, Bruckert E, Blondy P, Capeau J, Laville M, et al. Elevated levels of interleukin 6 are reduced in serum and subcutaneous adipose tissue of obese women after weight loss. J Clin Endocrinol Metab 2000;85:3338-42
27. Kern PA, Ranganathan S, Li C, Wood L, Ranganathan G. Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. American Journal of Physiology - Endocrinology & Metabolism 2001;280:E745-51
28. Wojtaszewski JF, Jorgensen SB, Frosig C, MacDonald C, Birk JB, Richter EA. Insulin signalling: effects of prior exercise. Acta Physiol Scand 2003;178:321-8
29. Febbraio MA, Pedersen BK. Muscle-derived interleukin-6: mechanisms for activation and possible biological roles. FASEB J 2002;16:1335-47
30. Carey AL, Steinberg GR, Macaulay SL, Thomas WG, Holmes AG, Ramm G, et al. Interleukin-6 increases insulin-stimulated glucose disposal in humans and glucose uptake and fatty acid oxidation in vitro via AMP-activated protein kinase. Diabetes 2006;55:2688-97
31. Yuen DY, Dwyer RM, Matthews VB, Zhang L, Drew BG, Neill B, et al. Interleukin-6 attenuates insulin-mediated increases in endothelial cell signaling but augments skeletal muscle insulin action via differential effects on tumor necrosis factor-alpha expression. Diabetes 2009;58:1086-95
32. Lagathu C, Yvan-Charvet L, Bastard JP, Maachi M, Quignard-Boulange A, Capeau J, et al. Long-term treatment with interleukin-1beta induces insulin resistance in murine and human adipocytes. Diabetologia 2006;49:2162-73
33. He J, Usui I, Ishizuka K, Kanatani Y, Hiratani K, Iwata M, et al. Interleukin-1alpha inhibits insulin signaling with phosphorylating insulin receptor substrate-1 on serine residues in 3T3-L1 adipocytes. Molecular endocrinology (Baltimore, Md) 2006;20:114-24
34. Jager J, Gremeaux T, Cormont M, Le Marchand-Brustel Y, Tanti JF. Interleukin-1beta-induced insulin resistance in adipocytes through down-regulation of insulin receptor substrate-1 expression. Endocrinology 2007;148:241-51
35. Dinarello CA. Proinflammatory cytokines. Chest 2000;118:503-8
36. Maedler K, Sergeev P, Ris F, Oberholzer J, Joller-Jemelka HI, Spinas GA, et al. Glucose-induced beta cell production of IL-1beta contributes to glucotoxicity in human pancreatic islets. J Clin Invest 2002;110:851-60

37. Juge-Aubry CE, Somm E, Chicheportiche R, Burger D, Pernin A, Cuenod-Pittet B, et al. Regulatory effects of interleukin (IL)-1, interferon-beta, and IL-4 on the production of IL-1 receptor antagonist by human adipose tissue. J Clin Endocrinol Metab 2004;89:2652-8
38. Meier CA, Bobbioni E, Gabay C, Assimacopoulos-Jeannet F, Golay A, Dayer JM. IL-1 receptor antagonist serum levels are increased in human obesity: a possible link to the resistance to leptin? J Clin Endocrinol Metab 2002;87:1184-8
39. Larsen CM, Faulenbach M, Vaag A, Volund A, Ehses JA, Seifert B, et al. Interleukin-1-receptor antagonist in type 2 diabetes mellitus. N Engl J Med 2007;356:1517-26
40. Fischer CP, Perstrup LB, Berntsen A, Eskildsen P, Pedersen BK. Elevated plasma interleukin-18 is a marker of insulin-resistance in type 2 diabetic and non-diabetic humans. Clin Immunol 2005;117:152-60
41. Bruun JM, Stallknecht B, Helge JW, Richelsen B. Interleukin-18 in plasma and adipose tissue: Effects of obesity, insulin resistance, and weight loss. European Journal of Endocrinology 2007;157:465-71
42. Hung J, McQuillan BM, Chapman CML, Thompson PL, Beilby JP. Elevated interleukin-18 levels are associated with the metabolic syndrome independent of obesity and insulin resistance. Arterioscler Thromb Vasc Biol 2005;25:1268-73
43. Kiortsis DN, Mavridis AK, Vasakos S, Nikas SN, Drosos AA. Effects of infliximab treatment on insulin resistance in patients with rheumatoid arthritis and ankylosing spondylitis. Ann Rheum Dis 2005;64:765-6
44. Rosenvinge A, Krogh-Madsen R, Baslund B, Pedersen BK. Insulin resistance in patients with rheumatoid arthritis: Effect of anti-TNFalpha therapy. Scand J Rheumatol 2007;36:91-6
45. Hotamisligil GS. Inflammation and metabolic disorders. Nature 2006;444:860-7
46. Feve B, Bastard JP. The role of interleukins in insulin resistance and type 2 diabetes mellitus. Nature Reviews Endocrinology 2009;5:305-11
47. Shulman GI. Cellular mechanisms of insulin resistance. J Clin Invest 2000;106:171-6
48. Trayhurn P, Wood IS. Adipokines: inflammation and the pleiotropic role of white adipose tissue. The British journal of nutrition 2004;92:347-55

49. Hosogai N, Fukuhara A, Oshima K, Miyata Y, Tanaka S, Segawa K, et al. Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation. Diabetes 2007;56:901-11
50. Pasarica M, Sereda OR, Redman LM, Albarado DC, Hymel DT, Roan LE, et al. Reduced adipose tissue oxygenation in human obesity: evidence for rarefaction, macrophage chemotaxis, and inflammation without an angiogenic response. Diabetes 2009;58:718-25
51. Minoguchi K, Tazaki T, Yokoe T, Minoguchi H, Watanabe Y, Yamamoto M, et al. Elevated production of tumor necrosis factor-alpha by monocytes in patients with obstructive sleep apnea syndrome. Chest 2004;126:1473-9
52. Yokoe T, Minoguchi K, Matsuo H, Oda N, Minoguchi H, Yoshino G, et al. Elevated levels of C-reactive protein and interleukin-6 in patients with obstructive sleep apnea syndrome are decreased by nasal continuous positive airway pressure. Circulation 2003;107:1129-34
53. Larsen JJ, Hansen JM, Olsen NV, Galbo H, Dela F. The effect of altitude hypoxia on glucose homeostasis in men. J Physiol 1997;504:241-9
54. Oltmanns KM, Gehring H, Rudolf S, Schultes B, Rook S, Schweiger U, et al. Hypoxia causes glucose intolerance in humans. Am J Respir Crit Care Med 2004;169:1231-7
55. Hodson L, Humphreys SM, Karpe F, Frayn KN. Metabolic signatures of human adipose tissue hypoxia in obesity. Diabetes 2013;62:1417-25
56. Hotamisligil GS. Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. Cell 2010;140:900-17
57. Basu S, Zethelius B, Helmersson J, Berne C, Larsson A, Arnlov J. Cytokine-mediated inflammation is independently associated with insulin sensitivity measured by the euglycemic insulin clamp in a community-based cohort of elderly men. Int J Clin Exp Med 2011;4:164-8
58. Londahl M, Katzman P, Nilsson A, Hammarlund C. Hyperbaric oxygen therapy facilitates healing of chronic foot ulcers in patients with diabetes. Diabetes Care 2010;33:998-1003
59. Clarke RE, Tenorio LMC, Hussey JR, Toklu AS, Cone DL, Hinojosa JG, et al. Hyperbaric Oxygen Treatment of Chronic Refractory Radiation Proctitis: A Randomized and Controlled Double-Blind Crossover Trial With Long-Term

Follow-Up. International Journal of Radiation Oncology Biology Physics 2008;72:134-43.e15

60. Wilkinson D, Doolette D. Hyperbaric oxygen treatment and survival from necrotizing soft tissue infection. Arch Surg 2004;139:1339-45
61. Thom SR. Hyperbaric oxygen: its mechanisms and efficacy. Plast Reconstr Surg 2011;127 Suppl 1:131S-41S
62. Ekanayake L, Doolette DJ. Effects of hyperbaric oxygen treatment on blood sugar levels and insulin levels in diabetics. SPUMS J 2001;31:16-20
63. Trytko B, Bennett MH. Blood sugar changes in diabetic patients undergoing hyperbaric oxygen therapy. SPUMS J 2003;33:62-9
64. Al-Waili NS, Butler GJ, Beale J, Abdullah MS, Finkelstein M, Merrow M, et al. Influences of Hyperbaric Oxygen on Blood Pressure, Heart Rate and Blood Glucose Levels in Patients with Diabetes Mellitus and Hypertension. Arch Med Res 2006;37:991-7
65. Rocco M, Antonelli M, Letizia V, Alampi D, Spadetta G, Passariello M, et al. Lipid peroxidation, circulating cytokine and endothelin 1 levels in healthy volunteers undergoing hyperbaric oxygenation. Minerva Anestesiol 2001;67:393-400
66. van den Blink B, van der Kleij AJ, Versteeg HH, Peppelenbosch MP. Immunomodulatory effect of oxygen and pressure. Comp Biochem Physiol 2002;Part A, Molecular & integrative physiology. 132:193-7
67. Fildissis G, Venetsanou K, Myrianthefs P, Karatzas S, Zidianakis V, Baltopoulos G. Whole blood pro-inflammatory cytokines and adhesion molecules post-lipopolysaccharides exposure in hyperbaric conditions. Eur Cytokine Netw 2004;15:217-21
68. Buras JA, Holt D, Orlow D, Belikoff B, Pavlides S, Reenstra WR. Hyperbaric oxygen protects from sepsis mortality via an interleukin-10-dependent mechanism. Crit Care Med 2006;34:2624-9
69. Kudchodkar B, Jones H, Simecka J, Dory L. Hyperbaric oxygen treatment attenuates the pro-inflammatory and immune responses in apolipoprotein E knockout mice. Clin Immunol 2008;128:435-41
70. Rinaldi B, Cuzzocrea S, Donniacuo M, Capuano A, Di Palma D, Imperatore F, et al. Hyperbaric oxygen therapy reduces the toll-like receptor signaling pathway in multiple organ failures. Intensive Care Med 2011;37:1110-9

71. Benson RM, Minter LM, Osborne BA, Granowitz EV. Hyperbaric oxygen inhibits stimulus-induced proinflammatory cytokine synthesis by human blood-derived monocyte-macrophages. Clin Exp Immunol 2003;134:57-62
72. Thom SR, Mendiguren I, Hardy K, Bolotin T, Fisher D, Nebolon M, et al. Inhibition of human neutrophil beta2-integrin-dependent adherence by hyperbaric O2. American Journal of Physiology - Cell Physiology 1997;272:C770-C7
73. Alex J, Laden G, Cale ARJ, Bennett S, Flowers K, Madden L, et al. Pretreatment with hyperbaric oxygen and its effect on neuropsychometric dysfunction and systemic inflammatory response after cardiopulmonary bypass: A prospective randomized double-blind trial. J Thorac Cardiovasc Surg 2005;130:1623-30
74. Yogaratnam JZ, Laden G, Guvendik L, Cowen M, Cale A, Griffin S. Hyperbaric oxygen preconditioning improves myocardial function, reduces length of intensive care stay, and limits complications post coronary artery bypass graft surgery. Cardiovasc Revasc Med 2010;11:8-19
75. Tiganis T. Reactive oxygen species and insulin resistance: the good, the bad and the ugly. Trends Pharmacol Sci 2011;32:82-9
76. Wilkinson D, Chapman IM, Heilbronn LK. Hyperbaric oxygen therapy improves peripheral insulin sensitivity in humans. Diabet Med 2012;29:986-9

CHAPTER 2

2a. Authorship statement

2b. Publication:

Wilkinson D, Chapman IM, Heilbronn LK. Hyperbaric oxygen therapy improves peripheral insulin sensitivity in humans. Diabet Med. 2012;29(8):986-9. doi: 10.1111/j.1464-5491.2012.03587.x. PubMed PMID: 22269009.

Statement of Authorship

Title of Paper	Hyperbaric oxygen therapy improves peripheral insulin sensitivity in humans
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Wilkinson D, Chapman IM, Heilbronn LK. Hyperbaric oxygen therapy improves peripheral insulin sensitivity in humans. Diabetic Medicine. 2012;29(8):986-9. doi: 10.1111/j.1464-5491.2012.03587.x

Principal Author

Name of Principal Author (Candidate)	David Wilkinson
Contribution to the Paper	Design of study, acquisition and interpretation of data, writing and revising manuscript
Overall percentage (%)	50%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 19/5/20

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Ian Chapman
Contribution to the Paper	Design of study, acquisition and interpretation of data, revising manuscript
Signature	Date 18/5/20

Name of Co-Author	Leonie Heilbronn
Contribution to the Paper	Acquisition and interpretation of data, writing and revising manuscript

Signature		Date	18/5/20

Please cut and paste additional co-author panels here as required.

DOI: 10.1111/j.1464-5491.2012.03587.x

Short Report: Pathophysiology

Hyperbaric oxygen therapy improves peripheral insulin sensitivity in humans

D. Wilkinson[1], I. M. Chapman[2] and L. K. Heilbronn[2,3]

[1]Hyperbaric Unit, Royal Adelaide Hospital, [2]Discipline of Medicine, The University of Adelaide, Adelaide and [3]Robinson Institute, North Adelaide, SA, Australia

Accepted 18 January 2012

Abstract

Aim Hyperbaric oxygen therapy is known to reduce fasting blood glucose in individuals with Type 2 diabetes. However, the mechanisms of this effect are not clear. The aim of this study was to determine whether peripheral insulin sensitivity by hyperinsulinaemic euglycaemic clamp is increased in patients presenting for hyperbaric oxygen therapy.

Methods Participants were non-obese individuals without Type 2 diabetes (n = 5) or obese patients with Type 2 diabetes (n = 5). Patients were given 100% oxygen at 2.0 absolute atmospheres for 2 h, six sessions per week for 5 weeks.

Results Peripheral insulin sensitivity was increased in the whole cohort (P = 0.04). Subsequent analysis revealed that this was significant at both treatment 3 (+37.3 ± 12.7%, P = 0.02) and treatment 30 (+40.6 ± 12.6%, P = 0.009). HbA_{1c} was significantly reduced in subjects without diabetes only ($P < 0.05$).

Conclusion Insulin sensitivity increased within 3 days of hyperbaric oxygen treatment and this was maintained for 30 sessions. This increase in insulin sensitivity is equivalent to that observed following moderate weight loss. The mechanisms underlying the insulin-sensitizing effect of hyperbaric oxygen require further elucidation.

Diabet. Med. 29, 986–989 (2012)

Keywords adipose tissue hypoxia, hyperbaric oxygen, insulin resistance, obesity

Abbreviation HOMA-IR, homeostasis model assessment of insulin resistance

Introduction

Uses of hyperbaric oxygen therapy include decompression sickness and to aid wound and ulcer healing in Type 2 diabetes mellitus. There is some evidence that hyperbaric oxygen reduces fasting blood glucose in patients with Type 2 diabetes [1,2] by at least 20% [2–4] and lowers HbA_{1c} and the inflammatory marker, C-reactive protein and fasting insulin resistance, as measured by homeostasis model assessment of insulin resistance (HOMA-IR) [3]. However, no studies have examined whether hyperbaric oxygen therapy changes peripheral insulin sensitivity as assessed by the gold standard, the hyperinsulinaemic euglycaemic clamp. It is also not clear whether the hyperbaric oxygen-induced reduction in blood glucose is unique to people with diabetes, or a physiological change for which individuals with Type 2 diabetes are unable to compensate. The aim of this study therefore was to determine whether peripheral insulin sensitivity is improved during hyperbaric oxygen treatment in humans, and whether this occurs in individuals with and without Type 2 diabetes.

Correspondence to: Leonie K. Heilbronn, The University of Adelaide—Department of Medicine, Level 6, Eleanor Harrald Building, Frome Rd, Adelaide 5005, Australia. E-mail: leonie.heilbronn@adelaide.edu.au

Subjects and methods

Patients

Non-obese individuals without Type 2 diabetes or obese males with Types 2 diabetes, aged 40–80 years, presenting for an initial assessment for hyperbaric oxygen therapy to the Hyperbaric Medical Unit of the Royal Adelaide Hospital were screened for participation. Indications for hyperbaric oxygen treatment included diabetic ulcer (n = 2), mandibular osteoradionecrosis (n = 6) and radiation proctitis (n = 2). All individuals with diabetes were receiving insulin therapy and three were also receiving oral hypoglycaemic agents; both were withheld on the mornings of the clamp. No medications were changed during the study. Participants were excluded if they

were inpatients, had recent or current active systemic infection, were using corticosteroids or had changes in insulin requirements within the last 3 months. The study was approved by the Human Ethics Committee and participants provided written consent.

Study design

Participants were studied in the Hyperbaric Unit on three occasions at 08.00 h after fasting from midnight the preceding night and were instructed not to change usual exercise habits over the course of the study. To assist this, patients were also provided with car parking facilities on site. Height and weight was recorded and two cannulae were placed intravenously in contralateral arm veins. Fasting blood samples were taken and the hyperinsulinaemic clamp (80 mU m^{-2} min^{-1}) was initiated with a variable infusion of 25% dextrose to clamp blood glucose levels at 6 mmol/l. The first 80 min of the clamp was performed in ambient air according to previously described techniques [5] and the last 2 h was performed either in ambient air at baseline, or during the 3rd and 30th exposure to hyperbaric oxygen treatment. Glucose infusion rate was calculated as the final 30 min of the clamp and adjusted for body surface area of the patient. Blood samples were assessed using a bedside glucose monitor (Accucheck Advantage; Roche Diagnostics, Castle Hill, NSW, Australia), using a glucose dehydrogenase method that is accurate when the sample oxygen level is high. The sample was also sent for later assessment in the laboratory. One blood sample was taken for measurement of steady-state insulin. The hyperbaric oxygen treatment schedule for study participants was identical to treatment received by patients not enrolled in the study. This involved daily treatment from Monday to Saturday (6 days/week) for 30 treatments. Each treatment lasted for 2 h and involved compression to 2 atmospheres absolute, breathing oxygen via a 'hood' system for 90 min with a 30-min decompression back to 1 atmosphere.

Biochemistry

Serum insulin was measured by electrochemiluminescence (Modular e170; Roche Diagnostics). C-peptide was measured using two-site chemiluminescence (Immulite 2000; Siemens, Bayswater, VIC, Australia). Glucose was measured using a hexokinase method (Olympus 4500; Beckman, Brea, CA, USA). HbA_{1c} was measured by cation exchange chromatography (Variant II; Bio-Rad Laboratories, Gladesville, NSW, Australia).

Statistical analysis

Analyses were performed using SPSS version 15.0 (SPSS Inc., Chicago, IL, USA) with one-way ANOVA or repeated-measures ANOVA, and subsequent testing of time points was performed using the paired *t*-test. $P < 0.05$ was considered significant and data are presented as mean ± SE, unless stated otherwise.

Results

Baseline patient characteristics are presented in Table 1. Individuals with Type 2 diabetes were heavier ($P < 0.001$). Fasting glucose and insulin were not different between groups, as there was high variation in Type 2 diabetes, but HbA_{1c} was significantly higher in individuals with Type 2 diabetes ($P = 0.007$). Peripheral insulin sensitivity was not statistically lower in those with Type 2 diabetes ($P = 0.1$).

Peripheral insulin sensitivity was significantly increased by hyperbaric oxygen therapy at three and 30 visits in the whole cohort ($P = 0.04$, Fig. 1a). Subsequent analysis revealed this was significant at both treatment 3 (+37.3 ± 12.7%, $P = 0.02$) and treatment 30 (40.6 ± 12.6%, $P = 0.009$). There was no group effect, although splitting the data set revealed that significance was reached only in those with Type 2 diabetes ($P = 0.008$), with four out of five individuals without diabetes improving insulin sensitivity ($P = 0.1$, Fig. 1b). HbA_{1c} was also significantly reduced in subjects without diabetes ($P = 0.05$, Fig. 1c), indicating a reduction in average blood glucose levels over the preceding weeks. However, fasting blood glucose (Fig. 1d) and insulin (data not shown) were not changed at treatment 3 or treatment 30 in either individuals with Type 2 diabetes or those without ($P = 0.4$ and $P = 0.2$, respectively). Steady-state insulin was higher in subjects with Type 2 diabetes (221 ± 41 vs. 120 ± 18 mU/µl, $P < 0.05$), as is often described in the literature [5], but this was not altered by hyperbaric oxygen treatment in either group (data not shown).

Discussion

This study is the first to show that insulin sensitivity is increased during hyperbaric oxygen therapy. This improvement is rapid, occurring within three treatments, and is sustained at least until the 30th treatment. The increase in insulin sensitivity was substantial, and similar in magnitude to the increase we have previously reported following 12% body weight loss at 1 year

Table 1 Patient characteristics

	No diabetes	Type 2 diabetes	*P*-value
n	5	5	
Age (years)	65 ± 4	66 ± 14	0.91
Weight (kg)	68.0 ± 12.4	91.0 ± 9.1	0.01
BMI (kg/m^2)	21.3 ± 2.9	31.3 ± 3.3	0.001
HbA_{1c} (mmol/mol) (%)	41 ± 3 (5.9 ± 0.3)	64 ± 14 (8.0 ± 1.2)	0.007
Glucose (mmol/l)	5.8 ± 1.0	9.9 ± 6.9	0.24
Insulin (mU/l)	7.3 ± 1.9	16.9 ± 5.0	0.15
C-peptide (nmol)	856 ± 226	725 ± 312	0.74

Means ± SD.

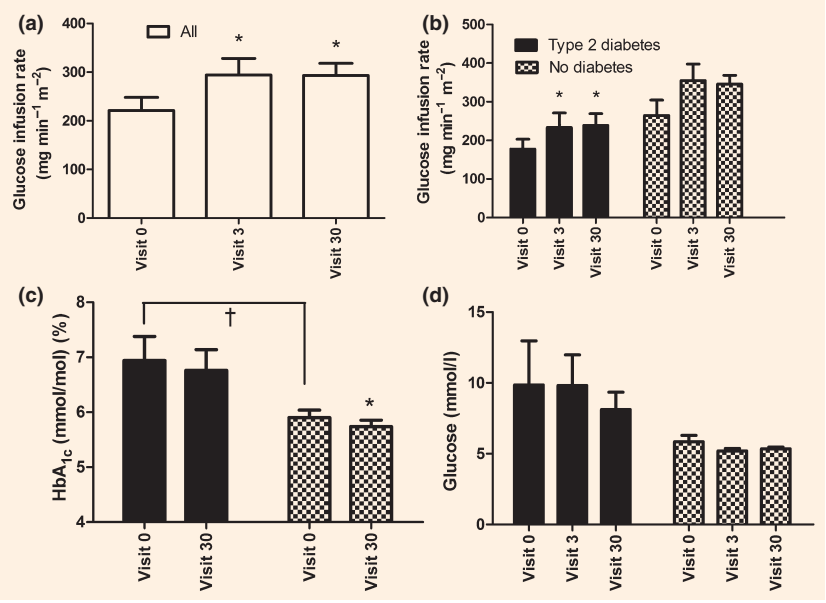


FIGURE 1 Insulin sensitivity at baseline (visit 0) and during the third (visit 3) and 30th (visit 30) treatment with hyperbaric oxygen therapy (a) in the whole cohort; or (b) grouped into individuals with and without Type 2 diabetes (Type 2 diabetes vs. no diabetes), (c) HbA_{1c} and (d) fasting glucose by group at baseline and during hyperbaric oxygen therapy. *$P < 0.05$ vs. baseline; †$P = 0.05$ vs. no diabetes.

in patients with Type 2 diabetes undergoing a diet and exercise programme [6].

Current lifestyle trends are characterized by low levels of physical activity and over consumption of energy-dense foods. This has led to a worldwide increase in the prevalence of obesity. as well as a number of metabolic disturbances, including the development of insulin resistance. This is defined as a relative impairment in the ability of insulin to exert its effects on glucose and lipid metabolism in target tissues (e.g. skeletal muscle, liver) and is considered one of the best predictors of the future development of Type 2 diabetes [7]. Despite this, the causes of insulin resistance in humans remain unclear. Recently, two studies have shown that fasting glucose is reduced in patients with Type 2 diabetes following hyperbaric oxygen therapy [3,4], with significant increases in HOMA-IR also reported [4]. In this study, despite the small sample size that was tested, we showed significant increases of approximately 40% in peripheral insulin sensitivity following hyperbaric oxygen treatment, providing a potential mechanism for the reductions in fasting glucose observed in past studies. This improvement was approximately twice that reported previously by HOMA-IR [4]. Whilst the improvement in peripheral insulin sensitivity only reached statistical significance in patients with Type 2 diabetes, four out of five individuals without diabetes also improved insulin sensitivity following hyperbaric oxygen treatment and had reductions in HbA_{1c}, suggesting this insulin-sensitizing effect was not confined to individuals with Type 2 diabetes only. This reduction in HbA_{1c}, in the absence of reduced fasting glucose, also supports improved insulin sensitivity, as it suggests there was reduced postprandial glucose. However, greater numbers of people should be tested and future studies should test healthy individuals without any associated illnesses that may impact the results. However, as the insulin-sensitizing effect was observed within 3 days and hyperbaric oxygen does not promote wound healing so rapidly, we do not believe it likely that changes in health were the cause of the improvements in insulin sensitivity observed.

The mechanisms underlying this improvement in peripheral insulin sensitivity were not tested and may provide novel insight into the pathogenesis of insulin resistance. During hyperbaric oxygen treatment, oxygen is delivered at high concentration under increased pressure, and the increase in insulin sensitivity observed may be attributable to either factor alone or a combination of both. Hyperbaric oxygen increases the oxygen delivery to blood and tissues by more than 10-fold, and thus we speculate that hyperbaric oxygen treatment may act to improve insulin sensitivity by reducing adipose tissue hypoxia and subsequently inflammation. It is increasingly recognized that obesity and Type 2 diabetes are characterized by adipose tissue dysfunction, including increased adipocyte size and local tissue hypoxia [8]. Adipose tissue hypoxia has been linked to endoplasmic reticulum stress and chronic low-grade inflammation, both of which directly inhibit the insulin signalling cascade [9,10]. Recently, C-reactive protein was shown to be reduced following hyperbaric oxygen therapy [3], indicating that chronic low-grade inflammation may be partially resolved. Unfortunately, blood samples were not stored for later assessment in this study and so we cannot test this response and whether it was different between individuals with and without Type 2 diabetes. There are also alternative mechanisms of action, as hyperbaric oxygen treatment may act

to improve insulin sensitivity by stimulating mitochondrial biogenesis. Increased expression of the master regulator of mitochondrial biogenesis, peroxisome proliferator activated receptor-1 alpha (PGC1α), has been shown following hyperbaric oxygen therapy at 1.25 atmospheres absolute and 36% oxygen in rats [11]. This may be important, as individuals with Type 2 diabetes and those with a family history of Type 2 diabetes have reduced PGC1α expression and reduced mitochondrial function [12–15], which has been linked to increased lipid accumulation and reduced insulin action in muscle [16]. However, this is also speculative and further research in this area is required.

In summary, hyperbaric oxygen treatment is associated with increased insulin sensitivity, with onset by 3 days and persistence for at least 5 weeks. The insulin-sensitizing effect of hyperbaric oxygen treatment is probably present in people without as well as with Type 2 diabetes, at least in this patient population. Unanswered questions remain, including the duration and cause of the effect. Further studies may provide novel strategies for preventing and treating insulin resistance.

Competing interests

Nothing to declare.

Acknowledgements

We would like to thank the patients who donated their time to participate in this study and the help of the nurses at the hyperbaric unit. The authors acknowledge support from the Royal Adelaide Hospital Research Committee Project Grant and from the Hyperbaric Health/Divers Alert Network Asia-Pacific Research Grant.

References

1 Springer R. The importance of glucometer testing of diabetic patients pre and post-dive. *Undersea Biomed Res* 1991; **18**: 1.
2 Ekanayake L, Doolette D. Effects of hyperbaric oxygen treatment on blood sugar levels and insulin levels in diabetics. *SPUMS J* 2001; **31**: 16–20.
3 Karadurmus N, Sahin M, Tasci C, Naharci I, Ozturk C, Ilbasmis S *et al.* Potential benefits of hyperbaric oxygen therapy on atherosclerosis and glycaemic control in patients with diabetic foot. *Endokrynol Pol* 2010; **61**: 275–279.
4 Al-Waili NS, Butler GJ, Beale J, Abdullah MS, Finkelstein M, Merrow M *et al.* Influences of hyperbaric oxygen on blood pressure, heart rate and blood glucose levels in patients with diabetes mellitus and hypertension. *Arch Med Res* 2006; **37**: 991–997.
5 Heilbronn LK, Rood J, Janderova L, Albu JB, Kelley DE, Ravussin E *et al.* Relationship between serum resistin concentrations and insulin resistance in non-obese, obese, and obese diabetic subjects. *J Clin Endocrinol Metab* 2004; **89**: 1844–1848.
6 Albu JB, Heilbronn LK, Kelley DE, Smith SR, Azuma K, Berk ES *et al.* Metabolic changes following a 1-year diet and exercise intervention in patients with type 2 diabetes. *Diabetes* 2010; **59**: 627–633.
7 Warram JH, Martin BC, Krolewski AS, Soeldner JS, Kahn CR. Slow glucose removal rate and hyperinsulinemia precede the development of type II diabetes in the offspring of diabetic parents. *Ann Intern Med* 1990; **113**: 909–915.
8 Heilbronn LK, Campbell LV. Adipose tissue macrophages, low grade inflammation and insulin resistance in human obesity. *Curr Pharm Des* 2008; **14**: 1225–1230.
9 Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* 1993; **259**: 87–91.
10 Hotamisligil GS. Inflammation and metabolic disorders. *Nature* 2006; **444**: 860–867.
11 Yasuda K, Adachi T, Gu N, Matsumoto A, Matsunaga T, Tsujimoto G *et al.* Effects of hyperbaric exposure with high oxygen concentration on glucose and insulin levels and skeletal muscle-fiber properties in diabetic rats. *Muscle Nerve* 2007; **35**: 337–343.
12 Kelley DE, He J, Menshikova EV, Ritov VB. Dysfunction of mitochondria in human skeletal muscle in Type 2 diabetes. *Diabetes* 2002; **51**: 2944–2950.
13 Petersen KF, Dufour S, Befroy D, Garcia R, Shulman GI. Impaired mitochondrial activity in the insulin-resistant offspring of patients with Type 2 diabetes. *N Engl J Med* 2004; **350**: 664–671.
14 Mootha VK, Lindgren CM, Eriksson K-F, Subramanian A, Sihag S, Lehar J *et al.* PGC-1α-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet* 2003; **34**: 267–273.
15 Patti ME, Butte AJ, Crunkhorn S, Cusi K, Berria R, Kashyap S *et al.* Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: potential role of PGC1 and NRF1. *Proc Natl Acad Sci USA* 2003; **100**: 8466–8471.
16 Turner N, Heilbronn LK. Is mitochondrial dysfunction a cause of insulin resistance? *Trends Endo Metab* 2008; **19**: 324–330.

CHAPTER 3

3a. Introduction

The findings from the pilot study encouraged further investigation, however more resources would be required. With the support of my supervisors and co-investigators, we found appropriate funding for the next step – a larger study that sought participants rather than patients and also made available a metabolic laboratory which allowed investigation of possible mechanisms of action for this insulin-sensitising effect of HBOT.

3b. Authorship statement

3c. Publication:

Wilkinson D, Nolting M, Mahadi MK, Chapman I, Heilbronn L. Hyperbaric oxygen therapy increases insulin sensitivity in overweight men with and without type 2 diabetes. Diving Hyperb Med. 2015;45(1):30-6. PubMed PMID: 25964036.

Statement of Authorship

Title of Paper	Hyperbaric oxygen therapy increases insulin sensitivity in overweight men with and without type 2 diabetes
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Wilkinson D, Nolting M, Mahadi MK, Chapman I, Heilbronn L. Hyperbaric oxygen therapy increases insulin sensitivity in overweight men with and without type 2 diabetes. Diving & Hyperbaric Medicine. 2015;45(1):30-6

Principal Author

Name of Principal Author (Candidate)	David Wilkinson		
Contribution to the Paper	Design of study, acquisition and interpretation of data, writing and revising manuscript		
Overall percentage (%)	33%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	15/6/2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Mirjam Nolting		
Contribution to the Paper	Design of study, acquisition and interpretation of data, writing and revising manuscript		
Signature		Date	29/05/2020

Name of Co-Author	Mohd Kaisan Mahadi		
Contribution to the Paper	Design of study, acquisition and interpretation of data, writing and revising manuscript		
Signature		Date	19/5/2020

Please cut and paste additional co-author panels here as required.

Name of Co-Author	Ian Chapman
Contribution to the Paper	Design of study, interpretation of data and revising manuscript
Signature	Date 15/6/20

Name of Co-Author	Leonie Heilbronn
Contribution to the Paper	Design of study, acquisition and interpretation of data and revising manuscript
Signature	Date 8/6/20

Hyperbaric oxygen therapy increases insulin sensitivity in overweight men with and without type 2 diabetes

David Wilkinson, Mirjam Nolting, Mohd Kaisan Mahadi, Ian Chapman and Leonie Heilbronn

Abstract

(Wilkinson D, Nolting M, Mahadi MK, Chapman I, Heilbronn L. Hyperbaric oxygen therapy increases insulin sensitivity in overweight men with and without type 2 diabetes. *Diving and Hyperbaric Medicine*. 2015 March;45(1):30-36.)

Aims: The onset of insulin resistance is an important metabolic event in the development of type 2 diabetes. For patients with type 2 diabetes, we recently showed that peripheral insulin sensitivity was increased during hyperbaric oxygen treatment (HBOT). This study aims to investigate whether this occurs in a non-patient population with and without type 2 diabetes, along with the mechanism of this effect.

Methods: Overweight and obese male participants were recruited from the community, 11 without and eight with type 2 diabetes. Insulin sensitivity was measured by the glucose infusion rate (GIR) during a hyperinsulinaemic euglycaemic clamp (80 $mU \cdot m^{-2} \cdot min^{-1}$) at baseline and during the third HBOT session. Monocyte chemo-attractant protein-1 (MCP-1), tumour necrosis factor-χ(TNF-χ) and interleukin-6 (IL-6) were measured in fasting serum and adipose tissue samples taken for their gene expression at baseline and immediately following four HBOT sessions. Additional fasting serum samples were collected during the first HBOT at 0, 60 and 120 minutes, and 24-hours after the last HBOT.

Results: In response to HBOT, GIR was increased by 29 ± 32% in those without (n = 10, P = 0.01), and by 57 ± 66% in those with type 2 diabetes (n = 7, P = 0.04). This increase was maintained for 30 minutes post HBOT. Reduced MCP-1 and TNF-χwere observed after HBOT, whereas IL-6 was increased only in individuals without diabetes and this correlated with the increase in insulin sensitivity (r^2 = 0.72, P = 0.004).

Conclusions: Peripheral insulin sensitivity was increased following HBOT in overweight or obese males with and without type 2 diabetes; this increase was maintained for at least 30 minutes post HBOT. Changes in inflammatory cytokines may partly explain this effect.

Key words

Endocrinology, hyperbaric oxygen, obesity, diabetes, inflammation, metabolism, hyperbaric research

Introduction

Hyperbaric oxygen treatment (HBOT) is defined as breathing 100% oxygen at a pressure greater than 101.3 kPa and is used clinically to treat a range of conditions including non-healing wounds.[1] When patients with type 2 diabetes undergo HBOT they sometimes report symptoms of hypoglycaemia, while studies have shown that fasting glucose levels are reduced by a greater amount during HBOT as compared to room air in patients with type 2 diabetes.[2,3] In a recent pilot study of hospital patients with type 2 diabetes who were receiving a prescribed course of HBOT for a medical condition, we showed that insulin sensitivity, as measured by the hyperinsulinaemic euglycaemic clamp technique, was increased during the third and the thirtieth HBOT sessions.[4] The mechanism was not investigated and it was unknown whether the insulin-sensitising effect was influenced by their medical conditions improving over time.

Insulin resistance is defined as a relative impairment in the ability of insulin to exert its effect on glucose metabolism in target tissues (e.g., skeletal muscle, liver) and is considered one of the best predictors of the future development of type 2 diabetes.[5] Obesity is also associated with insulin resistance,[6] and both obesity and type 2 diabetes are increasing in prevalence and have become major health issues globally. Obesity-related insulin resistance is closely associated with a chronic, low-grade inflammatory response within adipose tissue, characterised by immune cell infiltration, altered cytokine production and activation of inflammatory signalling pathways.[7] Pro-inflammatory cytokines linked to insulin resistance include tumour necrosis factor (TNF)-χ[8] monocyte chemo-attractant protein (MCP)-1,[9,10] interleukin (IL)-6[11,12] and members of the IL-1 family; IL-1, IL-1 receptor antagonist (IL-1ra) and IL-18.[13–15]

This study aims to determine whether the insulin-sensitising effect of HBOT can be demonstrated in a relatively healthy urban population including those with and without type 2 diabetes, whether the effect is still measurable after exit from the hyperbaric chamber and whether HBOT-induced changes in insulin resistance are associated with changes in pro-inflammatory cytokines in serum and adipose tissue known to be associated with insulin resistance.

Methods

The study received ethics approval from the University of Adelaide and the Royal Adelaide Hospital (approval no: 100615). All investigations were conducted in accordance with the Declaration of Helsinki and all subjects provided written informed consent.

SUBJECTS AND SCREENING

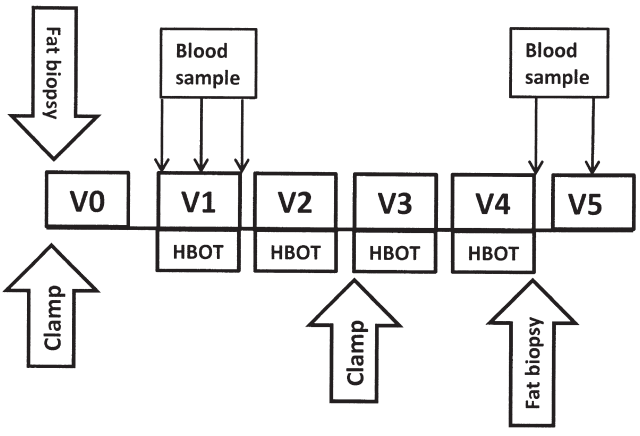
Advertisements and a web-recruitment company were

used to enlist overweight and obese male volunteers (BMI > 25kg·m^{-2}) who had no other excluded medical conditions apart from the sub-group with type 2 diabetes. As insulin sensitivity can vary throughout the menstrual cycle, only male volunteers were recruited. We undertook no specific investigation of the diabetes status of the volunteers, the diagnosis of type 2 diabetes was made from their personal medical history together with the prescription of appropriate medication. Excluded medical conditions included anything that could potentially alter insulin response or the inflammatory pathways being investigated, such as: smoking; consumption of more than three standard alcoholic drinks per day; vigorous exercise more often than twice a week; conditions that might be associated with a pathological inflammatory process or could influence inflammatory markers (such as sleep apnoea, malignancy, autoimmune and inflammatory diseases) and medication that might affect angiogenesis, lipid metabolism or have anti-inflammatory properties. Each volunteer was assessed for suitability to enter the hyperbaric chamber by a hyperbaric physician according to the standard clinical criteria used at the facility; this included history, examination and audiology assessment. Body composition was measured by dual-emission X-ray absorptiometry (DXA) to calculate fat mass and fat-free mass (FFM). Nineteen male volunteers were recruited, aged 45–70 years old, with BMI in the range of 24.3 to 45 kg·m^{-2}.

STUDY VISITS

Volunteers attended the Hyperbaric Medicine Unit at the Royal Adelaide Hospital on six occasions following a 10-hour overnight fast (Figure 1). Testing was undertaken at approximately the same time each morning and sampling was undertaken at a similar time each visit. Baseline assessments (V0) were performed one week and the following week participants attended the facility for five consecutive days (V1 to V5). Visits V1 to V4 included a routine 2-hour HBOT exposure. This involved compression to 203 kPa while breathing 100% oxygen for 90-minutes, followed by a linear decompression over 30 minutes and was administered in a rectangular twin-lock multiplace hyperbaric chamber (Fink Engineering/Cowan Engineering, Australia, 1994).

Figure 1
Timeline of study visits; V1–V5 were at same time of day on consecutive days; V0 was during the preceeding week



The 3.5-hour hyperinsulinaemic euglycaemic clamp was performed at baseline (V0) and visit V3. The baseline clamp was performed in normobaric room air, outside the hyperbaric chamber, as previously described.[16] Briefly, two intravenous cannulae were inserted into veins on opposite arms. One cannula was connected to an infusion of insulin (Actrapid®, Novo Nordisk, Baulkham Hills, Australia) at a fixed rate of 80 mU·m^{-2}·min^{-1}, together with a variable-rate infusion of 25% dextrose (Baxter Healthcare, Toongabbie, Australia). The other cannula allowed five to 10-minutely blood sampling to assess blood glucose levels by a hand-held glucometer (Accu-Chek Performa, Roche Diagnostics, Australia). The target blood glucose level was 6 mmol·L^{-1}. Insulin sensitivity was calculated from the glucose infusion rate (GIR) during two separate 30-minute steady-state (SS) periods at the end of the 3.5-hour clamp; SS1 corresponded with the period 2.5 to 3 hours and SS2 with 3 to 3.5 hours. The GIR was then standardized against FFM for each volunteer. The clamp was repeated during visit V3 with the two-hour HBOT session administered between the one- and the three-hour period of the clamp.

Therefore, when considering insulin sensitivity results, SS1 represented the last 30 minutes of the HBOT session while SS2 reflected the first 30 minutes immediately post-HBOT. Serum insulin was measured during both steady state periods. To avoid any physical effort that might influence glucose uptake, the volunteers remained sedentary in a chair which was wheeled in and out of the hyperbaric chamber. One non-diabetic subject was unable to adequately perform middle ear equalization during the first HBOT and took no further part in the study. Data from SS2 were not available for two volunteers.

Blood samples were taken at three time points during the first HBOT at visit V1: at time zero (pre-HBOT) and at 60 and 120-minutes relative to the 2-hour HBOT session. Further blood samples were taken at visit V4 (immediately after the fourth HBOT) and V5 (24 hours later). Blood samples were analysed for fasting glucose and insulin as well as cytokine markers of inflammation that are known to be associated with insulin resistance (TNF-χ IL-6, IL-18, IL-1ra and MCP-1). Abdominal subcutaneous adipose tissue was biopsied at baseline (V0) and visit V4 according to previously described techniques, snap frozen in liquid nitrogen and subsequently analysed for gene expression of inflammatory markers (IL-6, IL-1ra, TNF-χand MCP-1).[16]

LABORATORY ANALYSIS

Blood glucose samples sent to the laboratory were analysed by the hexokinase method (Olympus 4500, Beckman, USA) and insulin was measured by radioimmunoassay (Merck

Table 1
Baseline characteristics of men, stratified by diabetes status; median (95% CI), * $P \leq 0.001$

	Type 2 diabetes (n = 8)		**No diabetes** (n = 11)	
Age (years)	53	(49–60)	64	(53–66)
Height (m)	1.76	(1.69–1.79)	1.74	(1.69–1.80)
Weight (kg)	99.1	(87.9–111.5)	92.8	(80.4–108.5)
Body mass index (kg m^{-2})	30.8	(29.8–35.5)	30.5	(27.5–34.6)
Body fat (%)	35	(30–40)	32	(29–38)
Glucose (mmol·L^{-1})	9.8	(8.0–12.9) *	5.4	(5.0–5.9)
HDL (mmol·L^{-1})	1.2	(0.9–1.3)	1.2	(0.9–1.5)
LDL (mmol·L^{-1})	3.4	(2.1–4.2)	3.4	(2.1–4.2)
Triglycerides (mmol·L^{-1})	1.8	(0.8–4.9)	1.7	(1.0–2.0)
Total cholesterol (mmol·L^{-1})	5.6	(4.4–6.8)	5.3	(4.5–5.9)

Millipore, Billerica, MA, USA). Serum cytokine levels were determined using ELISA (R&D systems, Minneapolis, MN, USA). Total RNA was extracted from 100 mg adipose tissue using TRIzol reagent (Invitrogen, Carlsbad, CA). The integrity and concentration of RNA was assessed by spectrophotometry (Nanodrop, 2000, Thermoline). cDNA was synthesized using Omniscript RT kit (Qiagen, GmbH, Germany) and recombinant RNAsin ribonuclease inhibitor (Promega, Madison, WI) according to kit instructions. For RT-PCR analyses, we used gene-specific primer probes from Taqman (MCP-1, IL-6, TNF-χ IL1-ra) and Taqman universal PCR master mix (Applied Biosystems, Darmstadt, Germany). The samples were run in duplicate on an ABI Fast 7500 system (Applied Biosystems, Darmstadt, Germany) with internal negative controls and a standard curve. The cycle threshold (CT) value for each sample was normalized to the CT value of 18S ribosomal RNA to normalise for any changes in sample amplification, which was not different between V0 and V4.

STATISTICS

Statistical analysis was performed using SPSS for Windows (Version 19, SPSS Inc., Chicago, IL). Data were checked for normality by Shapiro-Wilk and log transformed prior to analysis if necessary. Differences between groups were analysed using one-way ANOVA. All other outcomes were analysed with linear mixed effects models using maximum likelihood estimation. Correlations were analysed by linear regression with coefficient of determination (r^2) and P value (Statistica v6, Statsoft, Tulsa, OK). Baseline characteristics, GIR and serum insulin were reported as median with 95% confidence intervals (CI_{95}). Significance was considered at $P < 0.05$.

Results

The baseline characteristics of groups stratified by diabetes status are shown in Table 1. Those with type 2 diabetes had higher fasting glucose ($P < 0.001$) and lower insulin sensitivity by hyperinsulinaemic clamp (Figure 2,

Figure 2
(A) Glucose infusion rate at baseline (V0) vs. HBOT (V3) during Steady State-1 (last 30 min of HBOT) in individuals with and without type 2 diabetes; (B), Glucose infusion rate at baseline vs. HBOT at Steady State-2 (first 30 min after HBOT) (mean and SEM, * $P < 0.05$, † $P < 0.01$)

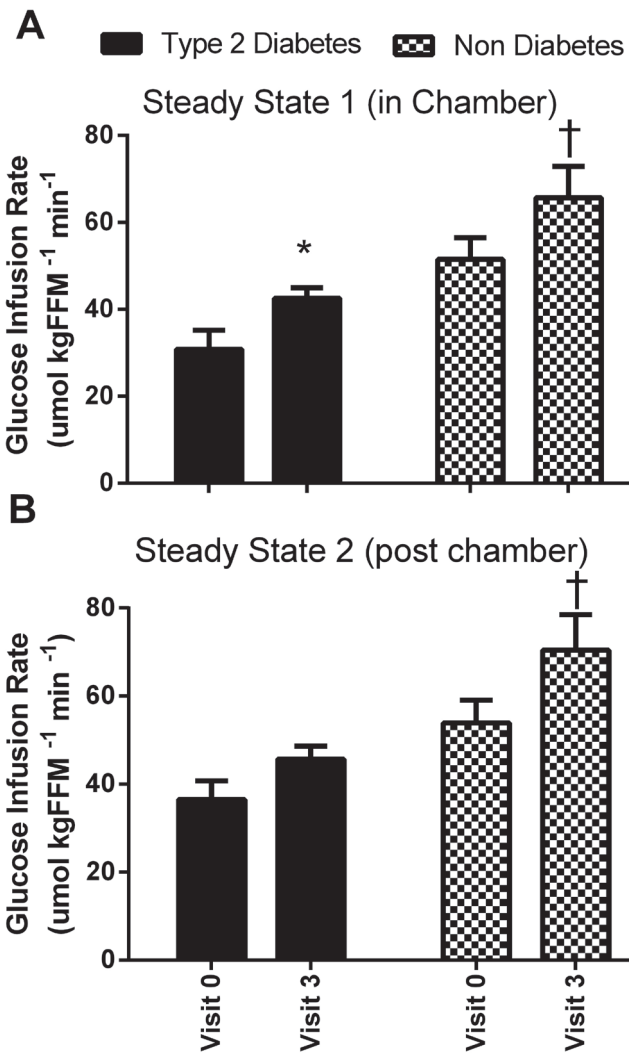


Figure 3

(A) Fasting glucose; (B) Insulin; (C); Monocyte chemotactic protein 1 – MCP-1; (D) Tumour necrosis factor – TNFχ (E) Interleukin–6 – IL-6 concentrations taken prior to and during the first HBOT exposure at 60 and 120 minutes, immediately following the 4th HBOT and 24 hours after the final HBOT (mean and SEM, * $P < 0.05$, † $P < 0.01$)

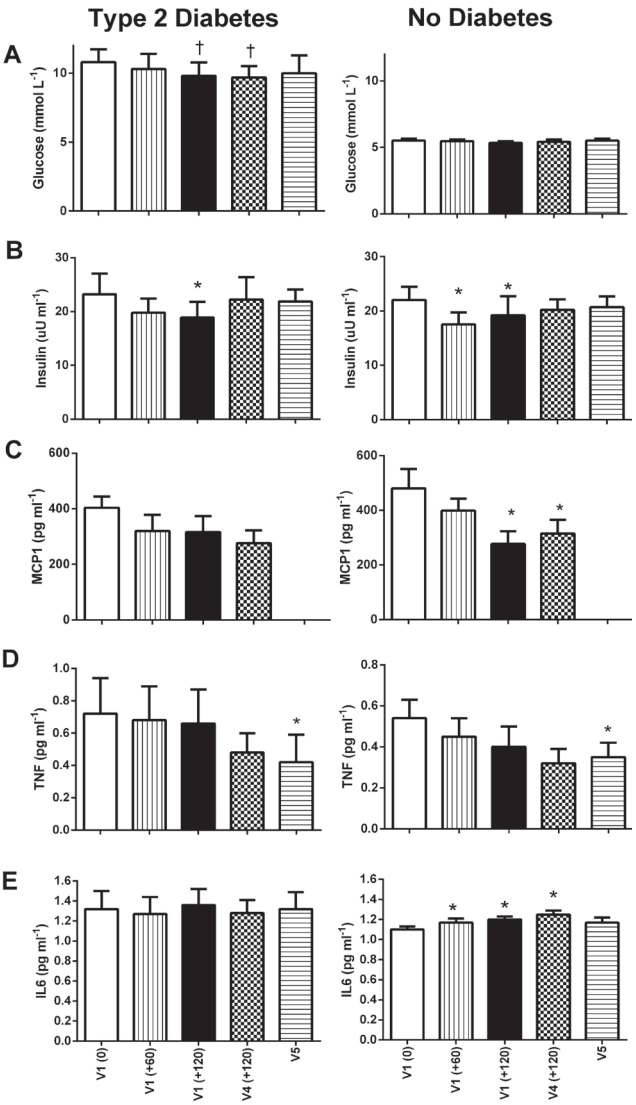
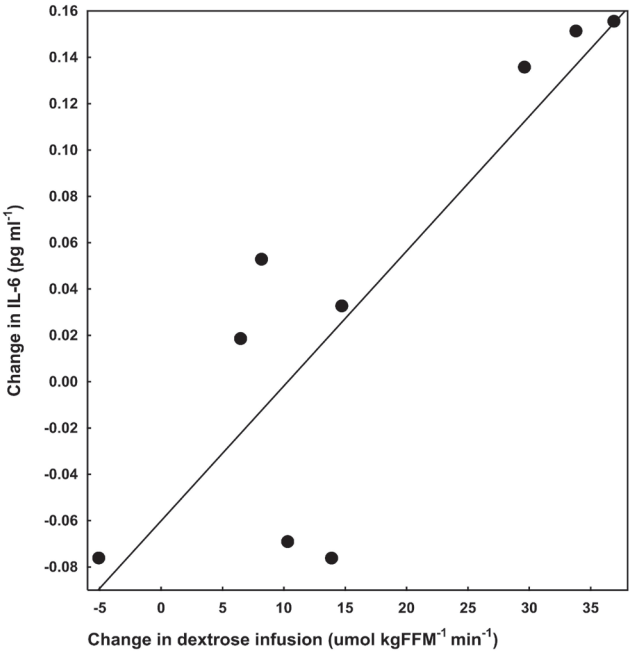


Figure 4

Relationship between the change in insulin sensitivity measured at visit V3 and change in serum interleukin–6 (IL-6) at visit V4 in non-diabetics ($r^2 = 0.72$, $P = 0.004$)



$P = 0.006$). A significant time effect was observed in the change in insulin sensitivity during the HBOT session (Figure 2A). For the group without diabetes, the median GIR at baseline in SS1 was 49.8 (39.6–62.7) μmol·kg·FFM^{-1}·min^{-1}. This increased during HBOT to 61.7 (49.4–82.1) μmol·kg·FFM^{-1}·min^{-1}. For the group with type 2 diabetes, baseline median GIR at SS1 was 32.6 (20.1–41.6) μmol·kg·FFM^{-1}·min^{-1}, increasing to 39.1 (36.6–48.5) μmol·kg·FFM^{-1}·min^{-1} during HBOT. The increase in insulin sensitivity was maintained for an additional 30 minutes after exit from the hyperbaric chamber whilst breathing normobaric air in those without diabetes ($n = 9$, $P = 0.008$, Figure 2B), but this was not significant in the group with diabetes ($n = 6$, Figure 2B). During the baseline hyperinsulinaemic euglycaemic clamp, steady state serum insulin was 204.3 (182.8–229.4) μU.ml^{-1} during SS1 and 199.2 (184.1–229.0) μU.ml^{-1} during SS2, with no significant difference during HBOT.

We observed significant time effects for the change in glucose, insulin, MCP-1, TNF-χand IL-6 with HBOT (all $P < 0.02$), with a time*group (diabetes/no diabetes) interaction observed in the change in fasting glucose only ($P = 0.03$). Further analysis by group revealed significant reductions in fasting glucose during the first and fourth HBOT sessions at 120 minutes only in those with type 2 diabetes (Figure 3A). Serum insulin was reduced during the first HBOT session in both groups (Figure 3B). MCP-1 was significantly reduced after HBOT at visits V1 and V4 in those without diabetes (Figure 3C), but this did not reach statistical significance in those with type 2 diabetes (Figure 3C). TNF-χ was significantly reduced 24-hours after the final HBOT in both groups (Figure 3D). In contrast, serum IL-6 was elevated in those without diabetes during and after HBOT at visits V1 and V4 (Figure 3E). The increase in IL-6 from baseline to visit 4 in the group without diabetes correlated with the increase in insulin sensitivity during SS2 ($n = 9$, $r^2 = 0.72$, $P = 0.004$, Figure 4). Neither group showed any significant changes for IL-1ra and IL-18 (data not shown). Adipose tissue was analysed for gene expression of IL-6, MCP-1, TNF-χand IL-1ra; however, no significant changes were detected (data not shown).

Discussion

In this study, we have demonstrated that peripheral insulin sensitivity is increased following HBOT in a relatively healthy urban population sample. Moreover, we have demonstrated that the increase in insulin sensitivity occurs in overweight and obese males without diabetes as well as those with type 2 diabetes. Importantly, the insulin sensitising effect was maintained after exit from the hyperbaric chamber for at least 30 minutes. We also observed small changes in inflammatory cytokines following HBOT that may have partly contributed to the observed increases in insulin sensitivity.

Diabetes is a common contributing or coincidental factor in patients referred for HBOT. Within hyperbaric medicine practice, it has been recognised for some time that patients with diabetes are prone to a fall in blood glucose during HBOT.[2,3] We also observed a significant fall in the blood glucose levels during the first HBOT in those with type 2 diabetes. Although greater decreases in fasting glucose inside versus outside the chamber have been reported,[2] we did not test this in our study and the changes could also be due to the prolonged length of the fast. Fasting glucose is predominantly under the control of hepatic glucose production; however, this was not specifically assessed in the current study. We also observed a fall in serum insulin during the first HBOT session in both groups; although other studies have found no effect of HBOT on insulin levels.[2,17] Our previous study tested a patient population during clinical HBOT exposure,[4] whilst the current study, which found a similar increase in insulin sensitivity, was in volunteers with no clinical indication for HBOT.

HBOT may induce an insulin-sensitizing effect by a number of possible mechanisms. Here, we studied circulating concentrations of pro-inflammatory cytokines since these have been observed in obesity and are closely associated with insulin resistance.[7] TNF-χis a pro-inflammatory cytokine which is overproduced from adipose tissue in human obesity,[8,18] and infusion of TNF-χinduces insulin resistance in humans.[19] The pro-inflammatory cytokine MCP-1 is also overproduced from adipose tissue in obesity[20] and impairs the insulin signalling cascade in a murine adipose tissue model independent of the associated macrophage infiltration.[9,10] Reductions in both TNF-χand MCP-1 were observed following HBOT and may partly explain the insulin-sensitizing effect, although the reduction in these cytokines did not correlate with the increase in insulin sensitivity.

IL-6 is a pleiotropic cytokine displaying both pro- and anti-inflammatory actions. Increased IL-6 is associated with human obesity and insulin resistance.[11,21] Conversely, exercise, a known insulin sensitiser, is associated with a transient release of IL-6 from muscle,[22] and acute infusion of IL-6 in humans leads to an increase in insulin sensitivity as measured by clamp studies.[12] IL-6 was not changed in those with type 2 diabetes, but was acutely increased by HBOT in those without diabetes. Interestingly, this was positively associated with increased insulin sensitivity. However, the changes in IL-6 are clinically small and may be a chance finding. We did not observe changes in IL-6 expression in adipose tissue, but no other tissues were investigated in this study.

The literature is mixed regarding the effect of HBOT on circulating cytokines, although most studies support an anti-inflammatory action of HBOT. Animal models suggest HBOT has, in part, an anti-inflammatory action in positive outcomes to abdominal sepsis,[23] multi-organ dysfunction[24] and development of atherosclerosis.[25] Human clinical data suggest HBOT-induced immunomodulation may be behind reduced restenosis following coronary angioplasty and stenting,[26] better outcome following cardio-pulmonary bypass,[27] and following ischaemia-reperfusion-related soft-tissue crush injury.[28] Even HBOT in the treatment of decompression illness is recognised to include an anti-inflammatory modulation of neutrophil activity as part of the therapeutic mechanism.[29] However, isolated cytokine changes should be interpreted with caution since the final effect on insulin sensitivity may depend on "*a subtle balance of their relative concentrations (high or low), kinetics (acute or chronic) and targets*".[15]

Alternatively, it has been proposed that insulin resistance may be induced by adipose tissue dysfunction secondary to hypoxia.[30] The growth of adipocytes in obesity is not matched by the blood supply, which may result in reduced oxygen delivery and regions of relative hypoxia.[30] Certainly, lower oxygen partial pressures have been measured in the adipose tissue of obese humans compared to lean controls.[31] However, another study concluded that adipose tissue had low oxygen consumption and the measurement of lactate/pyruvate ratios in blood draining this tissue revealed no evidence of metabolic stress.[32] The effects of hyperbaric oxygen on adipose tissue physiology have not been reported previously. However, studies investigating the reverse, using a hypoxic breathing gas mixture, have produced conflicting results. In two human studies using hyperinsulinaemic euglycaemic clamps, insulin resistance increased during acute exposure to hypoxia,[33] but decreased after a more chronic hypoxia protocol.[34] The substantial rise in tissue oxygen tensions associated with HBOT will also be accompanied by a transient increase in reactive oxygen species (ROS). This warrants further investigation since ROS, whilst having the potential to cause cell damage, also act as vital messengers in cell signalling,[35] including a positive effect on insulin signalling.[36]

This study employed the hyperinsulinaemic euglycaemic clamp which is considered to be the gold standard technique to assess peripheral insulin sensitivity.[37] Performing the clamp in a hyperbaric chamber was novel and required consideration of some technical issues and physiological

responses. Our glucometer used glucose dehydrogenase as the strip reagent, found to be more accurate than glucose oxidase when exposed to increased ambient oxygen.[38] Microvascular alterations in blood flow can influence measurement of insulin sensitivity as a consequence of varying the glucose delivery to the tissues.[39] Therefore, it is relevant to consider that vasoconstriction is an expected physiological response to hyperbaric oxygenation.[40] While the effects of HBOT on the microvasculature have not been tested, the sustained increase in insulin sensitivity observed upon exit from the hyperbaric chamber suggests our results were not influenced by changes in tissue blood flow.

Insulin resistance is a pivotal early change in obesity-related type 2 diabetes. The identification of pathways that influence insulin responsiveness may potentially lead to clinical therapies that prevent the development or progression of this disease. This study introduces a pathway that has not previously been exploited. The new findings, that HBOT can also increase insulin sensitivity in those without diabetes and also that the effect is sustained for a period after HBOT, have implications beyond diabetes involving obesity and glucose metabolism broadly. Further studies are now required to describe the precise mechanisms involved and to define the time course of the insulin sensitising effect – how much HBOT is required to initiate the effect and how long it persists after leaving the hyperbaric chamber.

Conclusions

This study has demonstrated that hyperbaric oxygen leads to an increase in insulin sensitivity in an overweight and obese male population with and without type 2 diabetes mellitus. Furthermore, the increase in insulin sensitivity was still evident 30 minutes after exiting the hyperbaric chamber. We have also demonstrated a favourable modulation of inflammatory markers in response to HBOT that may partly explain this effect on insulin sensitivity.

References

1 Gesell L, editor. *Hyperbaric oxygen therapy indications*. 12th ed. Durham, NC: Undersea and Hyperbaric Medical Society; 2008.

2 Ekanayake L, Doolette DJ. Effects of hyperbaric oxygen treatment on blood sugar levels and insulin levels in diabetics. *SPUMS Journal.* 2001;31:16-20.

3 Trytko B, Bennett MH. Blood sugar changes in diabetic patients undergoing hyperbaric oxygen therapy. *SPUMS Journal.* 2003;33:62-9.

4 Wilkinson D, Chapman IM, Heilbronn LK. Hyperbaric oxygen therapy improves peripheral insulin sensitivity in humans. *Diabet Med.* 2012;29:986-9.

5 Warram JH, Martin BC, Krolewski AS, Soeldner JS, Kahn CR. Slow glucose removal rate and hyperinsulinemia precede the development of type II diabetes in the offspring of diabetic parents. *Ann Intern Med.* 1990;113:909-15.

6 Hotamisligil GS. Inflammation and metabolic disorders. *Nature.* 2006;444:860-7.

7 Heilbronn LK, Campbell LV. Adipose tissue macrophages, low grade inflammation and insulin resistance in human obesity. *Curr Pharm Des.* 2008;14:1225-30.

8 Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science.* 1993;259:87-91.

9 Tateya S, Tamori Y, Kawaguchi T, Kanda H, Kasuga M. An increase in the circulating concentration of monocyte chemoattractant protein-1 elicits systemic insulin resistance irrespective of adipose tissue inflammation in mice. *Endocrinology.* 2010;151:971-9.

10 Kanda H, Tateya S, Tamori Y, Kotani K, Hiasa K, Kitazawa R, et al. MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *J Clin Invest.* 2006;116:1494-505.

11 Kern PA, Ranganathan S, Li C, Wood L, Ranganathan G. Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. *Am J Physiol Endocrinol Metab.* 2001;280:E745-51.

12 Carey AL, Steinberg GR, Macaulay SL, Thomas WG, Holmes AG, Ramm G, et al. Interleukin-6 increases insulin-stimulated glucose disposal in humans and glucose uptake and fatty acid oxidation in vitro via AMP-activated protein kinase. *Diabetes.* 2006;55:2688-97.

13 Jager J, Gremeaux T, Cormont M, Le Marchand-Brustel Y, Tanti JF. Interleukin-1beta-induced insulin resistance in adipocytes through down-regulation of insulin receptor substrate-1 expression. *Endocrinology.* 2007;148:241-51.

14 Lagathu C, Yvan-Charvet L, Bastard JP, Maachi M, Quignard-Boulange A, Capeau J, et al. Long-term treatment with interleukin-1beta induces insulin resistance in murine and human adipocytes. *Diabetologia.* 2006;49:2162-73.

15 Feve B, Bastard JP. The role of interleukins in insulin resistance and type 2 diabetes mellitus. *Nature Reviews Endocrinology.* 2009;5:305-11.

16 Heilbronn LK, Rood J, Janderova L, Albu JB, Kelley DE, Ravussin E, et al. Relationship between serum resistin concentrations and insulin resistance in nonobese, obese, and obese diabetic subjects. *J Clin Endocrinol Metab.* 2004;89:1844-8.

17 Chen SJ, Yu CT, Cheng YL, Yu SY, Lo HC. Effects of hyperbaric oxygen therapy on circulating interleukin-8, nitric oxide, and insulin-like growth factors in patients with type 2 diabetes mellitus. *Clin Biochem.* 2007;40:30-6.

18 Kern PA, Saghizadeh M, Ong JM, Bosch RJ, Deem R, Simsolo RB. The expression of tumor necrosis factor in human adipose tissue. Regulation by obesity, weight loss, and relationship to lipoprotein lipase. *J Clin Invest.* 1995;95:2111-9.

19 Plomgaard P, Bouzakri K, Krogh-Madsen R, Mittendorfer B, Zierath JR, Pedersen BK. Tumor necrosis factor-alpha induces skeletal muscle insulin resistance in healthy human subjects via inhibition of Akt substrate 160 phosphorylation. *Diabetes.* 2005;54:2939-45.

20 Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW Jr. Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest.* 2003;112:1796-808.

21 Bastard JP, Jardel C, Bruckert E, Blondy P, Capeau J, Laville M, et al. Elevated levels of interleukin 6 are reduced in serum and subcutaneous adipose tissue of obese women after weight loss. *J Clin Endocrinol Metab.* 2000;85:3338-42.

22 Febbraio MA, Pedersen BK. Muscle-derived interleukin-6: mechanisms for activation and possible biological roles. *FASEB Journal.* 2002;16:1335-47.

23 Buras JA, Holt D, Orlow D, Belikoff B, Pavlides S, Reenstra

WR. Hyperbaric oxygen protects from sepsis mortality via an interleukin-10-dependent mechanism. *Crit Care Med.* 2006;34:2624-9.
24 Rinaldi B, Cuzzocrea S, Donniacuo M, Capuano A, Di Palma D, Imperatore F, et al. Hyperbaric oxygen therapy reduces the toll-like receptor signaling pathway in multiple organ failures. *Intensive Care Med.* 2011;37:1110-9.
25 Kudchodkar B, Jones H, Simecka J, Dory L. Hyperbaric oxygen treatment attenuates the pro-inflammatory and immune responses in apolipoprotein E knockout mice. *Clin Immunol.* 2008;128:435-41.
26 Sharifi M, Fares W, Abdel-Karim I, Koch JM, Sopko J, Adler D. Usefulness of hyperbaric oxygen therapy to inhibit restenosis after percutaneous coronary intervention for acute myocardial infarction or unstable angina pectoris. *Am J Cardiol.* 2004;93:1533-5.
27 Alex J, Laden G, Cale ARJ, Bennett S, Flowers K, Madden L, et al. Pretreatment with hyperbaric oxygen and its effect on neuropsychometric dysfunction and systemic inflammatory response after cardiopulmonary bypass: A prospective randomized double-blind trial. *J Thorac Cardiovasc Surg.* 2005;130:1623-30.
28 Bouachour G, Cronier P, Gouello JP, Toulemonde JL, Talha A, Alquier P. Hyperbaric oxygen therapy in the management of crush injuries: a randomized double-blind placebo-controlled clinical trial. *Journal of Trauma, Injury, Infection and Critical Care.* 1996;41:333-9.
29 Martin JD, Thom SR. Vascular leukocyte sequestration in decompression sickness and prophylactic hyperbaric oxygen therapy in rats. *Aviat Space Environ Med.* 2002;73:565-9.
30 Trayhurn P, Wood IS. Adipokines: inflammation and the pleiotropic role of white adipose tissue. *Br J Nutr.* 2004;92:347-55.
31 Pasarica M, Sereda OR, Redman LM, Albarado DC, Hymel DT, Roan LE, et al. Reduced adipose tissue oxygenation in human obesity: evidence for rarefaction, macrophage chemotaxis, and inflammation without an angiogenic response. *Diabetes.* 2009;58:718-25.
32 Hodson L, Humphreys SM, Karpe F, Frayn KN. Metabolic signatures of human adipose tissue hypoxia in obesity. *Diabetes.* 2013;62:1417-25.
33 Oltmanns KM, Gehring H, Rudolf S, Schultes B, Rook S, Schweiger U, et al. Hypoxia causes glucose intolerance in humans. *Am J Respir Crit Care Med.* 2004;169:1231-7.
34 Lecoultre V, Peterson CM, Covington JD, Ebenezer PJ, Frost EA, Schwarz JM, et al. Ten nights of moderate hypoxia improves insulin sensitivity in obese humans. *Diabetes Care.* 2013;36:e197-8.
35 Thom SR. Hyperbaric oxygen: its mechanisms and efficacy. *Plast Reconstr Surg.* 2011;127(Suppl 1):131-41.
36 Tiganis T. Reactive oxygen species and insulin resistance: the good, the bad and the ugly. *Trends Pharmacol Sci.* 2011;32:82-9.
37 Muniyappa R, Lee S, Chen H, Quon MJ. Current approaches for assessing insulin sensitivity and resistance in vivo: advantages, limitations, and appropriate usage. *Am J Physiol Endocrinol Metab.* 2008;294:E15-26.
38 Tang Z, Louie RF, Lee JH, Lee DM, Miller EE, Kost GJ. Oxygen effects on glucose meter measurements with glucose dehydrogenase- and oxidase-based test strips for point-of-care testing. *Crit Care Med.* 2001;29:1062-70.
39 Rattigan S, Bussey CT, Ross RM, Richards SM. Obesity, insulin resistance, and capillary recruitment. *Microcirculation.* 2007;14:299-309.
40 Abel FL, McNamee JE, Cone DL, Clarke D, Tao J. Effects of hyperbaric oxygen on ventricular performance, pulmonary blood volume, and systemic and pulmonary vascular resistance. *Undersea Hyperb Med.* 2000;27:67-73.

Acknowledgements

We thank the participants and the staff of the Hyperbaric Medicine Unit, The Royal Adelaide Hospital who helped to perform this study.

Funding

The Royal Adelaide Hospital Foundation and Diabetes Australia Research Trust.

Conflicts of interest: nil

Submitted: 18 August 2014; revised 08 December 2014
Accepted: 14 January 2014

David Wilkinson[1,2], Mirjam Nolting[1], Mohd Kaisan Mahadi[2], Ian Chapman[2], Leonie Heilbronn[2]

[1] Hyperbaric Medicine Unit, Royal Adelaide Hospital, Adelaide, Australia
[2] Discipline of Medicine, The University of Adelaide, Adelaide, Australia

Address for correspondence:
David Wilkinson, FANZCA
Hyperbaric Medicine Unit, Royal Adelaide Hospital
North Terrace, Adelaide
South Australia 5000, Australia
Phone: *+61-(0)8-8222-5116*
Fax: *+61-(0)8-8232-4207*
E-mail: *<david.wilkinson@health.sa.gov.au>*

The database of randomised controlled trials in hyperbaric medicine maintained by Michael Bennett and his colleagues at the Prince of Wales Hospital Diving and Hyperbaric Medicine Unit, Sydney is at:
<http://hboevidence.unsw.wikispaces.net/>

Assistance from interested physicians in preparing critical appraisals is welcomed, indeed needed, as there is a considerable backlog. Guidance on completing a CAT is provided. Contact Associate Professor Michael Bennett: <m.bennett@unsw.edu.au>

CHAPTER 4

4a. Introduction

The insulin sensitivity results from the last study were consistent with the findings of the pilot study. Furthermore, changes in serum inflammatory cytokines for some of the important players in the chronic low grade inflammation story (TNF-α and MCP-1) suggested we might be on the right path.

The finding that the increase in insulin sensitivity could be demonstrated in those without diabetes convinced us to continue our line of investigation using participants without diabetes because it would be technically easier and avoid changing diabetic medication together with fasting in preparation for the glucose clamp technique.

The next goal was to investigate the timing of this insulin-sensitising effect – its onset and its duration. Previous studies had performed the glucose clamp during the third HBOT, partly in case some accumulated exposure to HBOT was required and also to give the participants the opportunity to practice their ear clearing technique prior to the day of running the glucose clamp in the chamber. So the next study we planned was to perform the glucose clamp during the first HBOT intervention. The study design required our participants to undertake two hyperinsulinaemic euglycaemic glucose clamps on consecutive days – the day of the first HBOT intervention and the day immediately prior. Although the glucose clamp technique was easy to manage in participants without diabetes and we had no specific issues during the study, on reflection I would not plan to perform two 3½ hour hyperinsulinaemic euglycaemic glucose clamps on consecutive days again. So when it came to investigating the duration of the insulin-sensitising effect, we recognised the need for a technique of assessing insulin sensitivity that might be easier for everyone concerned but could still be repeated on several occasions in a relatively short space of time. For this purpose, we chose a frequently sampled intravenous glucose tolerance test (FSIGT) using minimal model analysis. For this study, we reverted back to measuring insulin sensitivity during the third HBOT intervention, as this had provided reliable results so far, and then planned to repeat the FSIGT 24 hours later in room air.

The two techniques produced conflicting results in regard to insulin sensitivity. The subsequent publication focused on the insulin sensitivity results and discussion of the two techniques used. Although blood samples and adipose tissue biopsies were taken, they were not reported here.

4b. Authorship statement

4c. Publication:

Wilkinson D, Szekely S, Gue B, Tam CS, Chapman I, Heilbronn LK. Assessment of insulin sensitivity during hyperbaric oxygen therapy. Diving and Hyperbaric Medicine. (accepted for publication 18 March 2020)

Statement of Authorship

Title of Paper	Assessment of Insulin Sensitivity during Hyperbaric Oxygen Therapy
Publication Status	☐ Published ☑ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	David Wilkinson, Suzy Szekely, Brian Gue, Charmaine S Tam, Ian Chapman, Leonie K Heilbronn Diving and Hyperbaric Medicine. Manuscript DHM-2020-2/R1 RESUBMISSION - (618). Accepted for publication 18/03/2020

Principal Author

Name of Principal Author (Candidate)	David Wilkinson		
Contribution to the Paper	Design of study acquisition and interpretation of data, writing and revising manuscript		
Overall percentage (%)	50 %		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	19/08/2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis, and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Suzy Szekely		
Contribution to the Paper	Design of study, acquisition and interpretation of data, writing and revising manuscript		
Signature		Date	10/08/2020

Name of Co-Author	Brian Gue		
Contribution to the Paper	Design of study, acquisition and interpretation of data, writing and revising manuscript		
Signature		Date	5/8/2020

Please cut and paste additional co-author panels here as required

Name of Co-Author	Charmaine S Tam		
Contribution to the Paper	Interpretation of data and revising manuscript		
Signature		Date	22/7/20

Name of Co-Author	Ian Chapman		
Contribution to the Paper	Design of study, interpretation of data and revising manuscript		
Signature		Date	27 JULY 2020

Name of Co-Author	Leonie Heilbronn		
Contribution to the Paper	Design of study, acquisition and interpretation of data and revising manuscript		
Signature		Date	27/7/20

Assessment of Insulin Sensitivity during Hyperbaric Oxygen Therapy

David Wilkinson[1, 2], Suzy Szekely[1], Brian Gue[2], Charmaine S Tam[3], Ian Chapman[2], Leonie K Heilbronn[2]

[1] Hyperbaric Medicine Unit, Royal Adelaide Hospital, Adelaide, Australia
[2] Adelaide Medical School, The University of Adelaide, Adelaide, Australia
[3] Centre for Translational Data Science and Northern Clinical School, Sydney, Australia

Corresponding author:
Dr David Wilkinson
Hyperbaric Medicine Unit
Royal Adelaide Hospital
Port Road
Adelaide. SA 5000

Phone: 08 7074 0180
Email: david.wilkinson@sa.gov.au

Keywords:
Endocrinology, Hyperbaric research, Obesity, Metabolism, Physiology

Abstract

Introduction: Previous studies using a hyperinsulinaemic euglycaemic glucose clamp have demonstrated an increase in peripheral insulin sensitivity in men with and without Type-2 Diabetes Mellitus on the third and thirtieth Hyperbaric Oxygen Therapy (HBOT) session. In two studies using different techniques for assessment of insulin sensitivity, we investigated the onset and duration of this insulin-sensitising effect of HBOT.

Methods: Men who were obese or overweight but without diabetes were recruited. One study performed a hyperinsulinaemic euglycaemic glucose clamp ($80mU.m^{-2}.min^{-1}$) at baseline and during the first HBOT exposure (n=9). Data were analysed by paired t-test. The other study assessed insulin sensitivity by a frequently sampled intravenous glucose tolerance test (FSIGT) at three time points: baseline, during the third HBOT and 24-hours post-HBOT (n=9). Results were analysed by repeated-measures ANOVA.

Results: There was a significant 23% increase in insulin sensitivity by clamp measured during the first HBOT exposure. The FSIGT showed no significant changes in insulin sensitivity.

Conclusions: The hyperinsulinaemic euglycaemic glucose clamp demonstrated a significant increase in peripheral insulin sensitivity during a single, 2-hour HBOT session in a group of men who were obese or overweight but without diabetes. As an alternate technique for assessing insulin sensitivity during HBOT, the FSIGT failed to show any changes during the third HBOT and 24-hours later, however modification of the study protocol should be considered.

Introduction

While Hyperbaric Oxygen Therapy (HBOT) is not used to treat diabetes mellitus *per se*, it has been observed that when people with diabetes undergo HBOT they may experience a decrease in blood glucose levels (BGL), potentially inducing clinical hypoglycaemia.[1, 2] One study showed a substantial average BGL decrease of $3.5mmol.l^{-1}$ during a 2-hour HBOT session, with no change in serum insulin concentrations, suggesting an increase in insulin sensitivity as an underlying mechanism.[3]

Insulin resistance is defined as a relative impairment of the action of insulin on target tissues, particularly muscle and liver. The development of insulin resistance is the best predictor of those likely to develop type-2 diabetes mellitus (T2DM) in the future.[4] The inverse of insulin resistance is termed insulin sensitivity. In addition, obesity is strongly

associated with the development of insulin resistance and T2DM via activation of a chronic inflammatory state.[5]-The insulin resistance has effects on peripheral tissue glucose uptake as well as hepatic glucose production although an important effect is found in the peripheral tissues, specifically muscle.[6]

Of the many techniques available to assess insulin sensitivity, the hyperinsulinaemic euglycaemic glucose clamp is the gold standard.[7, 8] In a preliminary study of men (with and without T2DM) who were receiving a course of 30 HBOT sessions for medical indications, the glucose clamp technique revealed a substantial and significant increase in insulin sensitivity from baseline during their third (37% increase) and thirtieth (41% increase) HBOT sessions.[9] On subgroup analysis, this increase was significant only in those with T2DM, however numbers were small. A subsequent study, again using the glucose clamp technique, enrolled men who were obese or overweight (BMI>25kg.m^{-2}), with and without T2DM.[10] This study demonstrated significant increases in insulin sensitivity of similar magnitude during the third daily HBOT session in those with T2DM (57% increase) and without (29% increase). The increased insulin sensitivity was still measurable 30-minutes after exit from the hyperbaric chamber.

Unanswered questions include how quickly the insulin-sensitising effect of HBOT occurs, how long it persists and its underlying mechanisms. To investigate this, we planned to assess insulin sensitivity during the first HBOT using the hyperinsulinaemic euglycaemic glucose clamp. However, while the glucose clamp technique is accurate, it is labour intensive and made more complicated by being performed within a hyperbaric chamber under pressure. We therefore designed a further study to assess an alternate, technically easier method of assessing insulin sensitivity in the chamber, which if sufficiently accurate could be more easily used for repeated studies on the same participant. Having previously shown that the insulin-sensitising effect could be demonstrated in men without T2DM, we designed these studies using men who were obese or overweight (BMI>25 kg.m^{-2}) but without diabetes. This paper reports two studies: the use of the hyperinsulinaemic euglycaemic glucose clamp to test the effect on insulin sensitivity during the first HBOT session and secondly, the use of a frequently sampled intravenous glucose tolerance test (FSIGT) to assess insulin sensitivity during HBOT and after 24-hours.

Methods

Both studies were approved by the Human Research Ethics Committee of the Royal Adelaide Hospital (RAH121212a, RAH140321) and the University of Adelaide and entered on a trial registry site (NCT02009813, NCT02136615). Both studies were carried out in accordance with the Declaration of Helsinki. All participants provided written, informed consent.

PARTICIPANT SELECTION

Both studies enrolled participants via local advertisement and a web-based recruitment company. Only men were studied as insulin sensitivity can vary throughout the menstrual cycle. Other inclusion criteria included age over 18 years with no history of diabetes; participants were obese or overweight (body mass index >25 $kg.m^{-2}$). Exclusion criteria included prescribed or non-prescribed medication that may affect glucose homeostasis (e.g. corticosteroids); smoking; alcohol intake >140 grams/week; regular, high-intensity exercise (> twice per week); blood donation or involvement in any other study within the last 3 months. All participants were assessed for fitness to undertake HBOT by a hyperbaric physician.

HYPERINSULINAEMIC EUGLYCAEMIC GLUCOSE CLAMP STUDY DESIGN

The hyperinsulinaemic euglycaemic glucose clamp was first described by DeFronzo in 1979.[11] Insulin is infused at a constant rate that is above fasting levels, to stimulate glucose disposal in peripheral tissues but suppress hepatic glucose output. A variable dose glucose infusion is guided by regular blood sampling to measure BGL and "clamp" the BGL at a pre-determined level (in this case, 6$mmol.l^{-1}$). After running the infusions for a period of time, a steady-state can be reached where BGL and glucose infusion are stable. At this point, the glucose infusion rate (GIR) is equal to the glucose disposal rate. The GIR is a direct measure of whole body glucose disposal for a given level of hyperinsulinaemia.[8]

Ten participants were enrolled. A dual-emission x-ray absorptiometry scan (DXA) was performed at baseline for all participants to determine fat free mass (FFM). All participants attended the Hyperbaric Medicine Unit after overnight fasting (10-hours). Two IV cannulae were inserted into contralateral arms, one for the insulin and glucose infusions and the other for blood sampling. A primed insulin (Actrapid, Novo Nordisk,

Baulkham Hills, Australia) solution (80mU.m^{-2}.min^{-1}) was infused for 3½ hours as previously described.[10] Blood samples were taken at 5-10 minute intervals and BGL measured by glucometer (Accu-Chek Performa, Roche Diagnostics, Sydney, Australia). BGL was maintained at 6mmol.l^{-1} with a variable infusion of 25% Dextrose (Baxter Healthcare, Old Toongabbie, Australia). Insulin sensitivity was determined by the GIR during two separate but consecutive 30-minute steady state (SS) periods in the last hour of the infusion; SS1 corresponded with 2½-3 hours and SS2 with 3-3½ hours. The GIR was standardised for FFM from the DXA scan.

The following day, all participants returned after overnight fasting and the 3½ hour glucose clamp was repeated using the same protocol, this time overlayed with a 2-hour HBOT session. The twin-lock, multiplace hyperbaric chamber (Fink Engineering/Cowan Engineering, Australia, 1994) was compressed to 203 kPa followed by breathing 100% oxygen by mask or hood during 90 minutes at 203 kPa and a 30-minute linear decompression to 101.3 kPa. Insulin sensitivity was determined by the GIR during the same two SS periods, so SS1 coincided with the last 30-minutes of the 2-hour HBOT session and SS2 with the first 30-minutes after exit from the chamber. Statistical analyses were performed using Statistica (version 12, Statsoft, Tulsa, OK, USA). Paired t-test was used to compare GIR. Significance was considered at $p<0.05$.

FREQUENTLY SAMPLED INTRAVENOUS GLUCOSE TOLERANCE TEST STUDY DESIGN

An indirect measure of insulin sensitivity was developed by Bergman in 1979 using mathematical modelling of glucose and insulin data from an intravenous glucose tolerance test.[12] Following the glucose bolus, frequent measurement of blood glucose and insulin are made. The complex relationship between glucose and insulin in the disposal of glucose from the blood is built into pharmacokinetic models that are fit to the data. Parameters that provide best fit are derived. This includes insulin sensitivity (S_I), defined as fractional glucose disappearance per insulin concentration unit.[8] Other parameters include: glucose effectiveness (S_G), the ability of glucose to promote its own disposal; the acute insulin response to glucose (AIR_G) or first-phase insulin response; the disposition index (DI), a product of insulin sensitivity and insulin secretion, which is a constant. The mathematics to calculate these parameters has been packaged into a commercially

available software program. The FSIGT has shown reasonable correlation with the glucose clamp (r=0.54).[7]

Twelve participants were enrolled. On the first study day (Day 1) all participants attended the Hyperbaric Medicine Unit at the Royal Adelaide Hospital after an overnight (10-hour) fast. A baseline FSIGT was performed in room air with the participant resting in a chair outside of the hyperbaric chamber according to the following protocol. Two intravenous lines were inserted into contralateral forearms and blood taken for time zero. A glucose bolus was given into one of the cannulae at time zero over 1 minute. The weight-dependant bolus used 25% Dextrose (Baxter Healthcare, Old Toongabbie, Australia) at $300mg.kg^{-1}$ to a maximum dose of 120ml (30gm Dextrose). Blood sampling from the other IV line was performed at 2, 4, 6, 8, 10, 12, 14, 16, 19, 22, 25, 30, 40, 50, 60, 70, 90, 120, 150 and 180 minutes.

Each participant then underwent 3 HBOT sessions on consecutive days (Days 2-4), with compression to 203kPa breathing oxygen for 90-minutes and a 30-minute decompression. During the third HBOT session on Day 4, another FSIGT was performed using the same protocol as on Day 1. Compression of the chamber to 203kPa takes 7 minutes and time zero for the bolus Dextrose injection aligned with the start of oxygen breathing during the 90-min period at 203kPa. A further FSIGT was performed 24-hours later on Day 5, in air outside the hyperbaric chamber. The 3 FSIGTs were performed at a similar time of the day.

Blood samples taken at each of the time points were analysed for glucose and insulin. Insulin was measured by radioimmunoassay (Millipore, St. Charles, MO, USA). Glucose was measured using commercial enzymatic kits on a Beckman AU480 clinical analyser (Beckman Coulter, Brea, CA, USA). All samples for each subject were analysed within the same analytic run to minimise instrument variation. The glucose and insulin data were entered into the minimal model software (MINMOD Millennium, Pasadena, CA, USA) to derive insulin sensitivity and the other parameters. These measures were statistically analysed by repeated measures ANOVA using SPSS for Windows (Version 22, SPSS, Chicago, IL, USA). Significance was considered at $p<0.05$.

Results

HYPERINSULINAEMIC EUGLYCAEMIC GLUCOSE CLAMP

One participant sustained a minor middle ear barotrauma during compression at the start of the HBOT. He was removed from the hyperbaric chamber and excluded from the study. Characteristics of the nine remaining participants are shown in Table 1. The GIR data were normally distributed by Shapiro-Wilk and Kolmogorov-Smirnov tests. Figure 1A shows the GIR during SS1 (the last 30 minutes of the HBOT session). There was a significant increase in insulin sensitivity from Day 1 to Day 2, as measured by the GIR ($t=-2.89$, $df=8$, $p=0.02$). Figure 1B shows the GIR during SS2 (the first 30 minutes after leaving the chamber), the rise was not statistically significant ($t=-1.87$, $df=8$, $p=0.10$).

[Insert Table 1 and Figure 1 here]

	Mean ± SD
Age	47 ± 5.7
Height (cm)	176.4 ± 10.3
Weight (kg)	97 ± 15.1
BMI ($kg.m^{-2}$)	31.1 ± 3
DXA fat free proportion (%)	64.3 ± 0.1
Baseline insulin sensitivity ($mg.kgFFM^{-1}.min^{-1}$)	8.57 ± 3.02

Table 1: Demographics of participants (glucose clamp study, n=9). BMI: Body Mass Index, DXA: dual-emission x-ray absorptiometry scan.

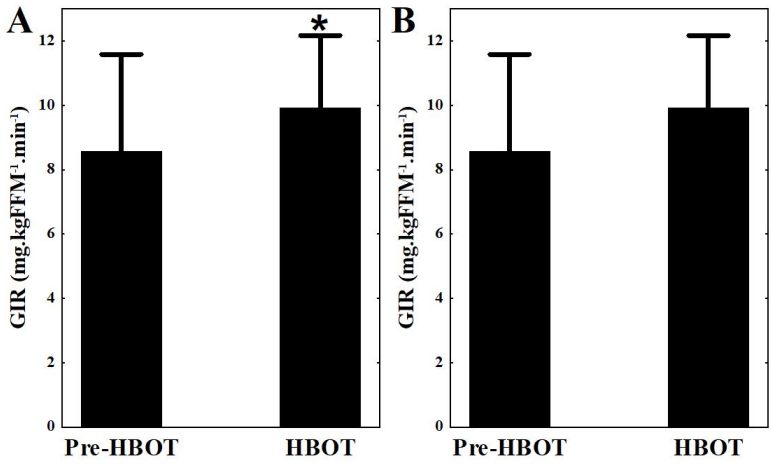


Figure 1: (A) Glucose infusion rate (GIR) at baseline vs HBOT during SS1 (last 30-minutes in chamber); (B) GIR at baseline vs HBOT during SS2 (first 30-minutes after HBOT). *p=0.02

FSIGT

One participant sustained a minor middle ear barotrauma at the start of compression and was removed from the hyperbaric chamber, another withdrew for personal reasons. Upon laboratory analysis, one further participant had glucose and insulin levels on arrival for the FSIGT on the third HBOT and again 24-hours later which suggested a failure to follow the fasting protocol, and these data were excluded. Characteristics of the remaining nine participants are shown in Table 2. The results of the minimal model analysis of the FSIGT are shown in Table 3. Data sets for all parameters showed large variances and there were no significant changes in any of the measured parameters.

[Insert Table 2 and Table 3 here]

	Mean ± SD
Age	37.1 ± 13
Weight (kg)	99.3 ± 15.2
Height (cm)	172.6 ± 3.8
BMI ($kg.m^{-2}$)	33.2 ± 4.1

Table 2: Demographics of participants (FSIGT study, n=9). BMI: Body Mass Index.

	Day 1	**Day 4**	**Day 5**
S_I ($mU.l^{-1}.min^{-1}$)	3.35 ± 1.27	3.82 ± 2.09	4.23 ± 3.38
S_G (min^{-1} x100)	1.55 ± 0.79	1.58 ± 0.92	1.48 ± 0.82
AIR_G ($mU.l^{-1}.min^{-1}$)	720 ± 462	573 ± 275	706 ± 364
DI	2304 ± 2004	1862 ± 1115	2165 ± 1089

Table 3: Insulin sensitivity and other parameters derived from minimal model analysis (mean ± SD). S_I: Insulin Sensitivity, S_G: Glucose effectiveness, AIR_G: Acute Insulin Response to Glucose, DI: Disposition Index.

Discussion

Using an in-chamber hyperinsulinaemic euglycaemic glucose clamp technique, we have previously shown that routine HBOT typically used for clinical indications is associated with significant increases from baseline in peripheral insulin sensitivity on the third day of daily HBOT sessions.[9, 10] Utilising the same clamp technique, we have now found that the HBOT-induced increase in insulin sensitivity occurs during the very first HBOT session. This study also confirms the previous findings that the insulin-sensitising effect

of HBOT can be identified in men without diabetes and is not specific to those with diabetes. The findings that the effect can be identified during the first HBOT exposure and in men without diabetes should make future studies examining the effects of HBOT on insulin sensitivity and the effects underlying them easier to undertake.

In our previous study using the clamp technique, HBOT significantly increased insulin sensitivity not only during the final 30-minutes of the 2-hours spent under HBOT conditions, but also during the first 30-minutes after exit from the hyperbaric chamber, when performed on the third HBOT exposure.[10] The current study used the glucose clamp technique on the first HBOT and found significantly increased insulin sensitivity under hyperbaric conditions (during Steady State 1). In contrast, there was not a significant increase over baseline insulin sensitivity during the first 30-minutes after leaving the chamber (Steady State 2). There is a trend towards an increase in insulin sensitivity, however small sample size and large variance in the data make statistical significance more difficult to achieve. Another consideration as to why Steady State 2 did not achieve significance in the current study could be that one HBOT has less impact than three – there was a 23% increase in insulin sensitivity during the first HBOT compared to a 29% increase in men without diabetes during the third HBOT.[10] There may be some accumulation of the HBOT-effect with repeated exposures, however its duration of effect is not known. It is clear however, that one 2-hour HBOT session is sufficient to see a change in insulin sensitivity. This finding is also consistent with clinical practice in hyperbaric medicine where anecdotally, people with diabetes have experienced a fall in their BGL during their first HBOT session.

Our previous studies performed the clamp on the third HBOT session for two reasons: to improve the chances of identifying an effect if some accumulated exposure was important and also to give the participant the opportunity to practice middle ear equalisation manoeuvres that are required during pressurisation of the hyperbaric chamber, prior to undergoing the glucose clamp procedure. While potential difficulty with ear equalisation was assessed during their initial medical review, middle ear barotrauma continues to be the most frequent complication associated with clinical HBOT (approximately 2%).[13] Indeed, one of our participants in this study had been established on his second glucose clamp with infusions of glucose and insulin when he was wheeled into the chamber only to find he couldn't satisfactorily equalise his ears at the start of the compression. This

required his removal from the hyperbaric chamber and from the study. Even with the small sample size in this study, a significant increase in insulin sensitivity was identified, consistent with the two previously published studies.

Our attempts to replace the glucose clamp technique with the simpler-to-use FSIGT however, have not been successful. While the FSIGT requires frequent blood sampling over several hours, it avoids the necessity of passing samples through the medical lock for immediate glucometer analysis and the rapid decisions required to maintain blood glucose concentrations during a glucose clamp. However, under the same HBOT conditions as in our three glucose clamp studies, all of which showed increased insulin sensitivity during the first or third HBOT session, we found no significant effect of HBOT on insulin sensitivity when assessed by the FSIGT during the third HBOT and at 24-hours later. There are a number of reasons the FSIGT may have failed to pick up such an effect. The sample size was small. The FSIGT is known to be less reliable in people with insulin resistance. Several modifications to this technique have been suggested, such as giving Tolbutamide or an insulin infusion early in the FSIGT, which has improved the correlation with glucose clamp studies.[7] However, in pursuit of a simpler technique and with a group of men without diabetes, we did not modify the FSIGT. Perhaps more likely, we performed the FSIGT too soon after the participants started their HBOT session. While we have demonstrated an increase in insulin sensitivity during steady state periods 2½ to 3½ hours into the clamp (at the end of an HBOT exposure), we have not specifically tested insulin sensitivity earlier in the HBOT session using a glucose clamp technique. If the insulin-sensitising effect of HBOT requires some duration of exposure to activate, then giving the glucose bolus of the FSIGT at the beginning of the HBOT session may not be the best time. The bulk of the glucose disposal would have taken place in the early part of the HBOT session and missed a later-onset effect identified in the clamp studies. Future studies using the FSIGT should perform the procedure towards the end of the HBOT session. On a cautionary note, such a study design may have the potential for the fasting participant with diabetes to develop hypoglycaemia during their HBOT session, prior to the FSIGT and they would need regular monitoring of their in-chamber BGL. If hypoglycaemia occurred during the HBOT, intervention would be required and the FSIGT would not be able to proceed. The third FSIGT performed 24-hours post HBOT also did not demonstrate an effect of HBOT on insulin sensitivity, but we cannot say whether this is because such an effect was not present (i.e. a stimulatory

effect of the previous day's HBOT had worn off), or whether such an effect was present but could not be detected due to limitations with the FSIGT technique.

The FSIGT was chosen because it was anticipated to be easier to perform and more easily tolerated by the participant than the glucose clamp. In the end, both techniques were found to be labour-intensive in a hyperbaric chamber. Importantly for undertaking assessment of insulin sensitivity in the novel environment of a hyperbaric chamber, every endeavour was made to perform these techniques according to established protocols. The fasting participants were tested at the same time of the day. They were kept sedentary in comfortable chairs for the duration of the study and wheeled into and out of the hyperbaric chamber. The glucometer utilised a glucose dehydrogenase reagent as opposed to glucose oxidase, making it less sensitive to ambient oxygen pressures.[14]

Our hyperbaric facility, along with many others, manages potential hypoglycaemia in patients with diabetes by monitoring their BGL before they enter the hyperbaric chamber and by repeating it if clinically indicated. Continued investigation is warranted in this field, both for the safety of hyperbaric patients with diabetes but also for the potential to identify novel pathways of glucose control.

Conclusion

The glucose clamp performed during the first HBOT session demonstrated a significant increase in insulin sensitivity, earlier than in our previously published studies which showed an increase in insulin sensitivity in men with and without diabetes on the third and thirtieth HBOT.[9, 10] The hyperinsulinaemic euglycaemic glucose clamp appears to be a useful tool to undertake these investigations. The FSIGT in its current design is probably not a good tool to assess insulin sensitivity in a hyperbaric chamber.

References

1. Trytko B, Bennett MH. Blood sugar changes in diabetic patients undergoing hyperbaric oxygen therapy. SPUMS J. 2003;33(2):62-9. PubMed PMID: 2003309266.
2. Al-Waili NS, Butler GJ, Beale J, Abdullah MS, Finkelstein M, Merrow M, et al. Influences of Hyperbaric Oxygen on Blood Pressure, Heart Rate and Blood

Glucose Levels in Patients with Diabetes Mellitus and Hypertension. Arch Med Res. 2006;37(8):991-7. doi: 10.1016/j.arcmed.2006.05.009. PubMed PMID: 17045116.

3. Ekanayake L, Doolette DJ. Effects of hyperbaric oxygen treatment on blood sugar levels and insulin levels in diabetics. SPUMS J. 2001;31(1):16-20. PubMed PMID: 32223833.
4. Warram JH, Martin BC, Krolewski AS, Soeldner JS, Kahn CR. Slow glucose removal rate and hyperinsulinemia precede the development of type II diabetes in the offspring of diabetic parents. Ann Intern Med. 1990;113(12):909-15. doi: 10.7326/0003-4819-113-12-909. PubMed PMID: 2240915.
5. Wellen KE, Hotamisligil GS. Inflammation, stress, and diabetes. J Clin Invest. 2005;115(5):1111-9. doi: 10.1172/JCI200525102. PubMed PMID: 18473870.
6. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: Direct role in obesity-linked insulin resistance. Science. 1993;259(5091):87-91. PubMed PMID: 7678183.
7. Borai A, Livingstone C, Ferns GA. The biochemical assessment of insulin resistance. Ann Clin Biochem. 2007;44(Pt 4):324-42. PubMed PMID: 17594780.
8. Muniyappa R, Lee S, Chen H, Quon MJ. Current approaches for assessing insulin sensitivity and resistance in vivo: advantages, limitations, and appropriate usage. Am J Physiol Endocrinol Metab. 2008;294(1):E15-26. Epub 2007/10/25. doi: 10.1152/ajpendo.00645.2007. PubMed PMID: 17957034.
9. Wilkinson D, Chapman IM, Heilbronn LK. Hyperbaric oxygen therapy improves peripheral insulin sensitivity in humans. Diabet Med. 2012;29(8):986-9. doi: 10.1111/j.1464-5491.2012.03587.x. PubMed PMID: 22269009.
10. Wilkinson D, Nolting M, Mahadi MK, Chapman I, Heilbronn L. Hyperbaric oxygen therapy increases insulin sensitivity in overweight men with and without type 2 diabetes. Diving Hyperb Med. 2015;45(1):30-6. PubMed PMID: 25964036.
11. DeFronzo RA, Tobin JD, Andres R. Glucose clamp technique: a method for quantifying insulin secretion and resistance. Am J Physiol. 1979;237(3):E214-23. Epub 1979/09/01. PubMed PMID: 382871.
12. Bergman RN, Ider YZ, Bowden CR, Cobelli C. Quantitative estimation of insulin sensitivity. Am J Physiol. 1979;236(6):E667-77. PubMed PMID: 443421.

13. Camporesi E. Side Effects. In: Weaver LK, editor. Hyperbaric Oxygen Therapy Indications. 13th ed. Florida: Best Publishing Company; 2014. p. 247-52.
14. Tang Z, Louie RF, Lee JH, Lee DM, Miller EE, Kost GJ. Oxygen effects on glucose meter measurements with glucose dehydrogenase- and oxidase-based test strips for point-of-care testing. Crit Care Med. 2001;29(5):1062-70. doi: 10.1097/00003246-200105000-00038. PubMed PMID: 11378622.

Acknowledgements

We thank the staff of the Hyperbaric Medicine Unit who helped perform these studies. I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

Funding

Grants provided by Royal Adelaide Hospital Research Committee and Australasian Diving and Hyperbaric Medicine Research Trust.

Conflicts of interest

Nil

CHAPTER 5

5a. Introduction

The last publication described two different techniques for measuring insulin sensitivity and reported the results using these techniques during an HBOT intervention. Both studies also took blood samples and adipose tissue biopsies pre and post-HBOT for further investigation of possible mechanisms of action for an insulin-sensitising effect of HBOT based on the changes in inflammatory cytokines identified earlier. These blood and adipose tissue results were not included in the last publication, but have been analysed in a separate report written in manuscript style. They are unpublished.

The literature review focused on an anti-inflammatory role for HBOT and the evidence for HBOT influencing inflammatory cytokine release and subsequent inflammatory processes involving ischaemia and sepsis, but is several years old now. Reviewing the cytokine data from these two studies revealed considerable inconsistency in the cytokine responses to HBOT. This provided the incentive to revisit the evidence, update the literature review in this respect and consider more recent evidence for HBOT having an impact on inflammatory cytokine release.

5b. Authorship statement

5c. Manuscript:

Inflammatory cytokine production and adipose tissue gene expression in response to Hyperbaric Oxygen – unpublished data

David C. Wilkinson, Bo Liu, Ian M. Chapman, Leonie K. Heilbronn

Statement of Authorship

Title of Paper	Inflammatory cytokine production and adipose tissue gene expression in response to Hyperbaric Oxygen – unpublished data
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	

Principal Author

Name of Principal Author (Candidate)	David Wilkinson		
Contribution to the Paper	Design of study, acquisition and interpretation of data, writing and revising manuscript		
Overall percentage (%)	50%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	06/09/2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Bo Liu		
Contribution to the Paper	Acquisition and interpretation of data, writing and revising manuscript		
Signature		Date	31/08/2020

Name of Co-Author	Leonie Heilbronn

Contribution to the Paper	Design of study, acquisition and interpretation of data and revising manuscript		
Signature		Date	31/8/2020

Please cut and paste additional co-author panels here as required.

Name of Co-Author	Ian Chapman		
Contribution to the Paper	Design of study, interpretation of data and revising manuscript		
Signature		Date	8/9/20

Inflammatory cytokine production and adipose tissue gene expression in response to Hyperbaric Oxygen – unpublished data

David C. Wilkinson[1, 2]
Bo Liu[2]
Ian M. Chapman[2]
Leonie K. Heilbronn[2]

[1] Hyperbaric Medicine Unit, Royal Adelaide Hospital, Adelaide, Australia
[2] Adelaide Medical School, The University of Adelaide, Adelaide, Australia

Abstract

Introduction: Obesity is associated with the release of inflammatory cytokines from adipose tissue creating a chronic low grade inflammatory state which is associated with insulin resistance. Hyperbaric oxygen therapy (HBOT) is widely considered to have anti-inflammatory actions. We report two studies which measured changes in serum inflammatory cytokines and adipose tissue gene expression in response to HBOT.

Methods: Men aged 18 years or older who were overweight or obese were recruited to either a study using a hyperinsulinaemic euglycaemic glucose clamp (n = 9) or a frequently sampled intravenous glucose tolerance test (FSIGT, n = 9). They underwent two or three HBOT interventions involving compression to 203 kPa (2 atmospheres absolute) for 90 minutes breathing oxygen via a hood, followed by a 30-minute decompression. The different study designs meant that blood sampling was not synchronised. The clamp technique study sampled blood at baseline, at both the start and end of the second HBOT and 24 hours later and were analysed for inflammatory cytokines (IL-6, gp130, TNF-α, MCP-1, CRP and HSP-70). The FSIGT study sampled blood at baseline, at the end of the first and second HBOT and 24 hours after the third HBOT and were analysed for IL-6, gp130 and CRP. Abdominal adipose tissue biopsies were taken for gene expression analysis prior to HBOT and immediately after two HBOT interventions. Changes in serum cytokines and gene expression data were analysed by Wilcoxon Matched Pair Test. Given the time differences in sampling between studies, results are reported separately.

Results: Compared to baseline, the glucose clamp study demonstrated a within group increase in IL-6 at the start ($P = 0.021$) and the end ($P = 0.008$) of the second HBOT. Serum gp130 was decreased at the end of the second HBOT ($P = 0.02$). The FSIGT study demonstrated no change in IL-6 but a within group decrease in soluble gp130 at the end of the second HBOT ($P = 0.038$) as well as 24 hours after the third HBOT ($P = 0.044$). Gene expression from the clamp technique study revealed a decrease in MMP9 after two HBOT ($P = 0.038$).
Conclusions: Serum IL-6 was increased and the related glycoprotein, soluble gp130, was decreased after HBOT in men who were overweight or obese. The increase in IL-6 found in the glucose clamp study was associated with a significant increase in insulin sensitivity. Further research should investigate the origin of the increased IL-6 and its association with an insulin-sensitising effect of HBOT.

Introduction

Metabolic research has identified that increased adipose tissue associated with obesity is a trigger for macrophage infiltration and activation.[1] The subsequent systemic release of inflammatory cytokines, including tumour necrosis factor α (TNF-α), monocyte chemo-attractant protein 1 (MCP-1) and interleukin 6 (IL-6) creates a chronic low grade inflammatory state that has been associated with the development of insulin resistance, diabetes, cardiovascular disease and some cancers.[2]

In a series of publications, we have demonstrated an insulin-sensitising effect of hyperbaric oxygen therapy (HBOT) using the hyperinsulinaemic euglycaemic glucose clamp. First, we demonstrated a significant increase in insulin sensitivity in a group of patients with and without diabetes during their third and thirtieth HBOT.[3] Subsequently, we demonstrated a significant increase in insulin sensitivity in a group of men who were overweight or obese, with and without diabetes during their third HBOT.[4] This study also found a favourable decrease in serum inflammatory cytokines in response to HBOT, namely reductions in serum levels of TNF-α and MCP-1.[4]

Two further studies have been published, however they reported only the primary study outcome of the measurement of insulin sensitivity under hyperbaric conditions.[5] One study used an hyperinsulinaemic euglycaemic glucose clamp technique while the other study used a frequently sampled intravenous glucose tolerance test (FSIGT). Both studies

also had blood samples and adipose tissue biopsies taken, which are now being reported here. While the participant characteristics of these two studies were generally similar, the timing of the blood samples – determined by the insulin sensitivity test scheduling – varied between studies. The results must therefore be considered separately.

The aim of this study was to investigate any change in serum inflammatory cytokines or in the expression of genes involved in adipose tissue inflammation and remodelling for a group of men who were overweight or obese following HBOT; and further, if any changes were associated with an insulin-sensitising effect of HBOT.

Methods

Both studies were approved by the Human Research Ethics Committee of the Royal Adelaide Hospital (RAH121212a, RAH140321) and the University of Adelaide and entered on a trial registry site (NCT02009813, NCT02136615). Both studies were carried out in accordance with the Declaration of Helsinki. All participants provided written, informed consent.

PARTICIPANTS

The participants were the same as those reported in the publication investigating techniques to measure insulin sensitivity under hyperbaric conditions.[5] Briefly, they were men who were overweight or obese (body mass index >25 $kg.m^{-2}$), aged over 18 years with no history of diabetes or other significant medical conditions. The clamp technique study recruited ten participants; one participant who suffered a minor ear barotrauma on his first HBOT was excluded from the study. The FSIGT study recruited twelve participants; one suffered a minor ear barotrauma during HBOT, one withdrew for personal reasons and another was excluded when laboratory data revealed a failure to follow fasting instructions. Both studies report the results from nine participants.

STUDY DESIGN

Participants underwent an overnight fast prior to each study day. The primary outcome for both studies – measurement of insulin sensitivity – was undertaken during the first HBOT intervention in the clamp technique study and during the third HBOT in the FSIGT study and have been previously reported.[5] Blood sampling for inflammatory cytokines was not possible on the days of insulin sensitivity measurement and so the

timing of the samples was not synchronised for both studies. The clamp technique study had bloods taken for baseline several days prior to HBOT intervention, then at the start of the second HBOT (i.e. 24 hours after the first HBOT) and the end of the second HBOT and then 24 hours later. Blood was analysed for inflammatory related cytokines TNF-α, MCP-1, IL-6, C reactive protein (CRP), glycoprotein 130 (gp130) and heat shock protein 70 (HSP-70). Baseline bloods were taken immediately prior to HBOT in the FSIGT study and then immediately after the first and the second HBOT interventions. The 24 hour blood sample in the FSIGT study was taken after having had three HBOT interventions. Blood was analysed for IL-6, gp130 and CRP. Abdominal subcutaneous adipose tissue was biopsied at baseline and after two HBOT interventions for both studies, according to previously described techniques,[6] snap frozen in liquid nitrogen and subsequently analysed for gene expression related to a range of genes involved with macrophage activity (CD40, CD68, CD163), angiogenesis (VEGFα, ANGPT2) and extracellular matrix remodelling (COL3A1, COL6A1, MMP2, MMP9, TIMP1).

LABORATORY ANALYSIS

Serum cytokines were measured using commercial ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Total RNA was extracted from ~ 100 mg sample of adipose tissue using TRI Reagent (Sigma, St. Louis, USA) following the manufacturer's instructions as described previously.[7] The concentration and purity of RNA were assessed by NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, CA,USA). cDNA synthesis was conducted using T100 Thermal Cycler (Bio-Rad, CA, USA) with 1000 ng of each RNA sample using the QuantiTect reverse transcription kit (Qiagen, Valencia, CA,USA) according to kit instructions. Quantitative real-time PCR was performed using the Taqman primers (Table 1) for macrophage (CD40, CD68, CD163), angiogenesis (VEGFα, ANGPT2) and extracellular matrix (COL3A1, COL6A1, MMP2, MMP9, TIMP1) and Fast Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The samples were run in duplicate on an ABI 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA) with internal negative controls and a standard curve (pooled from each participant). Relative gene expression was analysed using the $2^{-\Delta CT}$ method and

Gene symbol	Gene name	Assay ID
Housekeeping		
ACTB	Actin beta	Hs01060665_g1
Macrophage		
CD68	CD68 molecule	Hs02836816_g1
CD40	CD40 molecule	Hs01002913_g1
CD163	CD163 molecule	Hs00174705_m1
Angiogenesis		
VEGFα	vascular endothelial growth factor α	Hs00900055_m1
ANGPT2	Angiopoietin 2	Hs01048042_m1
Extracellular matrix		
COL3A1	Collagen type III alpha 1	Hs00943809_m1
COL6A1	Collagen type VI alpha 1	Hs01095585_m1
MMP2	Matrix metallopeptidase 2	Hs01548727_m1
MMP9	Matrix metallopeptidase 9	Hs00234579_m1
TIMP1	TIMP metallopeptidase inhibitor 1	Hs00171558_m1

Table 1: Taqman primers used for gene expression analysis.

standardised for housekeeping gene (ACTB) which was not different between baseline and post-HBOT.

STATISTICAL ANALYSIS

Statistical analyses were performed using Statistica (version 12, Statsoft, Tulsa, OK, USA). Pre-post changes in serum cytokines and gene expression were analysed by Wilcoxon Matched Pairs Test for each study separately. Significance was considered at $P < 0.05$.

Results

In the glucose clamp study we found a significant increase in serum IL-6 in response to HBOT (Figure 1A). Compared to baseline, IL-6 was increased at the start of the second HBOT (24 hours after the first HBOT, $P = 0.021$) as well as at the end of the second HBOT where mean levels more than doubled (Table 2, $P = 0.008$). However, IL6 levels had returned to basal levels 24 hours after the second treatment. There was no change in IL6 after 1 or 2 HBOT interventions, or 24 hours after the third HBOT in the FSIGT study (Figure 1B, Table 3).

In the glucose clamp study, we also found a significant decrease in serum soluble gp130 from baseline as compared to the end of the second HBOT (Figure 2A, $P = 0.02$), but gp130 was not different from basal levels compared to 24 hours after the first HBOT (Pre-HBOT #2) or 24 hours after the second HBOT. In the FSIGT study, there was also a reduction in soluble gp130 in response to HBOT (Figure 2B). Compared to baseline, soluble gp130 was reduced at the end of the second HBOT ($P = 0.038$) and at 24 hours after the third HBOT ($P = 0.044$).

There was no change in serum concentration of other cytokines measured in the clamp technique study (Table 2) or the FSIGT study (Table 3).

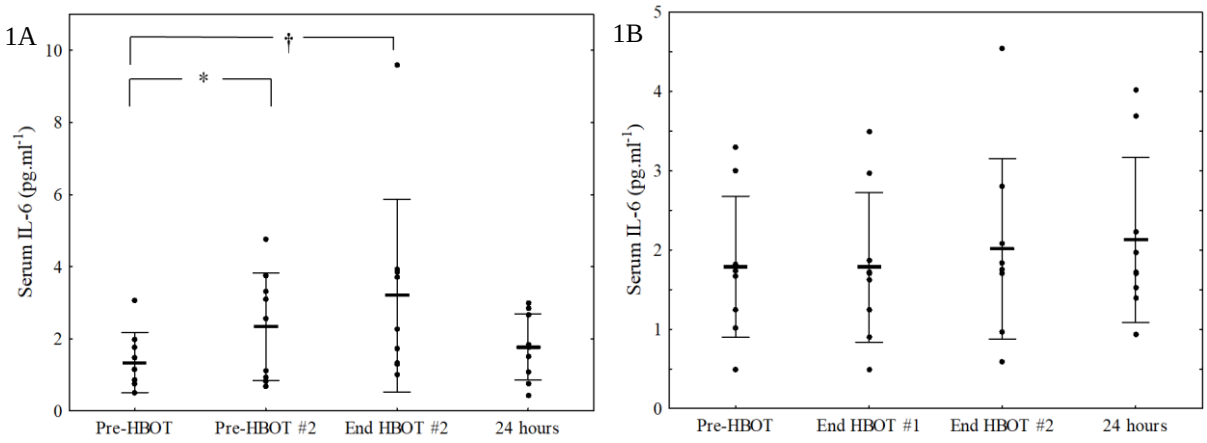


Figure 1: Serum IL-6 concentrations from **1A:** the glucose clamp study showed IL-6 increased from baseline compared to 24 hours after one HBOT (Pre-HBOT #2) and immediately after the second HBOT. (* $P = 0.021$, † $P = 0.008$) **1B:** The FSIGT study showed no change in IL-6 after one or two HBOT, or 24 hours after the third HBOT.

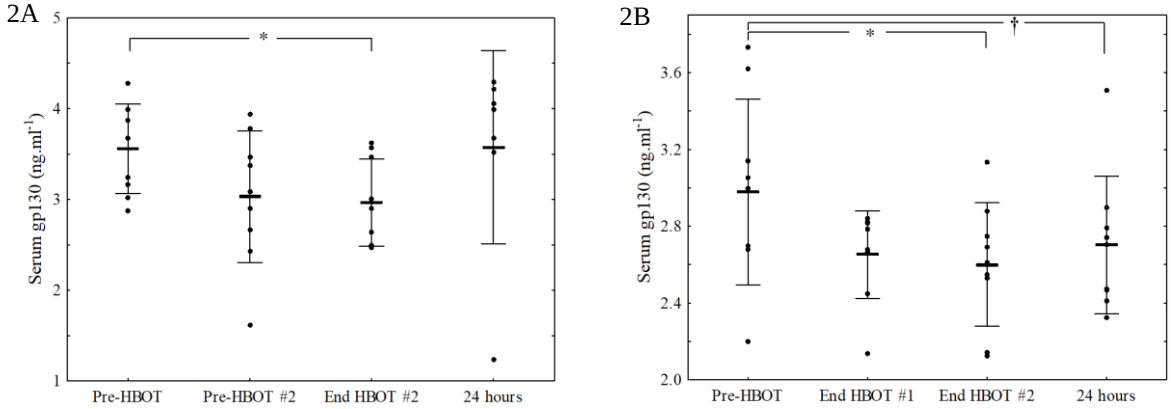


Figure 2: Serum soluble gp130 from **2A:** the glucose clamp study showed gp130 decreased from baseline compared to the end of the second HBOT. (* $P = 0.02$) **2B:** The FSIGT study showed gp130 decreased from baseline compared to the end of the second HBOT and 24 hours after the third HBOT (* $P = 0.038$, † $P = 0.044$)

	Pre-HBOT	Pre-HBOT #2	Post-HBOT #2	24 hours post-HBOT #2
IL-6 ($pg.ml^{-1}$)	1.345 (0.836)	2.341 (1.492) *****	3.198 (2.675) †	1.773 (0.919)
Gp130 ($ng.ml^{-1}$)	3.558 (0.492)	3.029 (0.724)	2.963 (0.481) ‡	3.572 (1.064)
CRP ($mg.l^{-1}$)	2.24 (1.76)	2.69 (1.9)	2.59 (1.9)	3.84 (2.73)
TNF-α ($pg.ml^{-1}$)	1.832 (0.787)	1.520 (0.803)	1.831 (0.434)	1.718 (0.274)
MCP-1 ($pg.ml^{-1}$)	102.59 (31.33)	79.71 (48.3)	99.91 (39.18)	102.65 (45.18)
HSP-70 ($ng.ml^{-1}$)	0.048 (0.006)	0.057 (0.009)	0.055 (0.01)	0.05 (0.01)

Table 2: Serum inflammatory cytokines in the glucose clamp study, expressed as mean (SD). IL-6 was increased before and after the second HBOT. Gp130 was decreased after the second HBOT. (* $P = 0.021$, † $P = 0.008$, ‡ $P = 0.02$)

	Pre-HBOT	Post-HBOT #1	Post-HBOT #2	24 hours post-HBOT #3
IL-6 ($pg.ml^{-1}$)	1.788 (0.889)	1.78 (0.942)	2.014 (1.138)	2.129 (1.041)
Gp130 ($ng.ml^{-1}$)	2.98 (0.48)	2.651 (0.228)	2.6 (0.323) *****	2.702 (0.358) †
CRP ($mg.l^{-1}$)	3.29 (1.97)	3.44 (2.18)	3.62 (2.79)	3.88 (2.79)

Table 3: Serum inflammatory cytokines in the FSIGT study, expressed as mean (SD). Gp130 was decreased after the second HBOT and 24 hours after the third HBOT. (* $P = 0.038$, † $P = 0.044$)

mRNA foldchange	Clamp study (*n*=8)		FSIGT study (*n*=9)	
	Pre-HBOT	Post-HBOT	Pre-HBOT	Post-HBOT
CD68	1.62 (0.58)	1.24 (0.4)	1.01 (0.93)	0.53 (0.28)
CD40	2.1 (2.0)	2.0 (1.43)	0.61 (0.44)	0.75 (0.65)
CD163	1.19 (0.48)	1.11 (0.36)	1.01 (0.45)	1.06 (0.41)
VEGFα	1.87 (0.93)	1.97 (1.23)	0.91 (0.51)	1.06 (0.87)
ANGPT2	0.004 (0.006)	0.005 (0.008)	-	-
COL3A1	1.13 (0.32)	1.42 (0.96)	0.94 (0.31)	0.96 (0.39)
COL6A1	1.33 (0.32)	1.3 (0.31)	0.82 (0.18)	0.78 (0.17)
MMP2	1.82 (0.41)	1.44 (0.38)	0.72 (0.27)	0.73 (0.27)
MMP9	1.71 (1.65)	0.61 (0.56) *****	2.07 (2.73)	0.52 (0.38)
TIMP1	4.02 (3.14)	2.46 (1.21)	0.57 (0.6)	0.61 (0.74)

Table 4: mRNA foldchange for genes analysed in both studies, expressed as mean (SD). There was a significant decrease in expression of MMP9 in the clamp technique study. (* $P = 0.036$).

Analysis of gene expression data from adipose tissue samples in the glucose clamp study revealed a significant decrease in expression of MMP9 after 2 HBOT interventions (Table 4, $P = 0.036$). There was no change in expression for any of the genes analysed in the FSIGT study.

Discussion

The two studies have shown that men who were overweight or obese have reduced serum soluble gp130 levels following two HBOT interventions. One of the studies – the glucose clamp study – found serum IL-6 was increased after HBOT. This study also reported a significant increase in insulin sensitivity following HBOT in this same group of men when measured using a hyperinsulinaemic euglycaemic glucose clamp.[5] These results should be considered in the context of an earlier study where we also found an acute increase in serum IL-6 and a significant increase in insulin sensitivity after HBOT intervention in a similar group of men.[4]

IL-6 is produced by many cell types although about one third of basal IL-6 secretion comes from macrophages within adipose tissue.[8] It has traditionally been considered a pro-inflammatory cytokine and there is considerable evidence to support this. Women with obesity were found to have increased IL-6 levels and reduced insulin sensitivity as measured by the fasting insulin resistance index.[9] Weight loss led to a reduction in IL-6 and an improvement in insulin sensitivity. Another study found that people with obesity had increased IL-6 levels in serum and adipose tissue, but it was serum IL-6 that was found to be best associated with reduced insulin sensitivity as measured by the intravenous glucose tolerance test.[10] Furthermore, IL-6 is one of the cytokines implicated in the chronic low grade inflammatory state associated with obesity and the subsequent development of metabolic disease.[2] However, other evidence indicates a far more complex role for IL-6 where it has also been shown to have an anti-inflammatory role. Acute intravenous infusion of IL-6 in a group of men without diabetes resulted in improved glucose disposal during a hyperinsulinaemic euglycaemic glucose clamp.[11] IL-6 is also released by skeletal muscle. During physical exercise, more IL-6 is released from muscle and it becomes the predominant source of serum IL-6 with serum concentrations increasing by up to 100 times.[12] At the same time, exercise itself has been shown to be an insulin sensitiser.[13] That IL-6 can elicit such widely variable responses – from pro-

inflammatory to anti-inflammatory – indicates considerable nuance in its action. A number of factors have been recognised to influence the IL-6 response including: whether the raised IL-6 is chronic or acute and its actual concentration, the source of the IL-6 and its target tissue as well as other classes of cytokines and co-factors that can modulate IL-6 activity in a hierarchical fashion.[14, 15] When IL-6 reaches target tissue, it can signal via two pathways, termed "trans" or classical signalling.[15-17] Activation of the "trans" signalling pathway enables IL-6 to interact with a very broad range of tissues, eliciting a generalised inflammatory response which is implicated in the chronic low grade inflammatory state. The classical signalling pathway restricts serum IL-6 to bind to only a few tissue types (hepatocytes and some white cells) with a specific and modulated response. Glycoprotein gp130 is a major factor in determining which pathway IL-6 activates. Gp130 is a ubiquitously expressed transmembrane receptor for the IL-6 family of cytokines. A small amount of gp130 detaches from membranes and exists as soluble gp130 in serum.[15] Here it appears to buffer serum IL-6 levels along with the action of other co-factors.[16] For example, a serum IL-6 complex that has already been primed to activate the "trans" signalling pathway by binding to a soluble IL-6 receptor, will bind preferentially with soluble gp130 in the serum and in doing so only permit activation of the classical pathway. Reducing the level of "trans" signalled IL-6 will reduce the pro-inflammatory characteristics of IL-6 action.[17]

The two current studies have demonstrated a significant and acute reduction in serum soluble gp130 in response to HBOT, but only one study also demonstrated a significant increase in serum IL-6. This increase in IL-6 in the glucose clamp study was also associated with an increase in insulin sensitivity in response to HBOT using a hyperinsulinaemic euglycaemic glucose clamp.[5] In an earlier study of men who were overweight or obese but without diabetes, we also found a significant increase in serum IL-6 following HBOT as well as an increase in insulin sensitivity using the clamp technique.[4] This earlier study also took adipose tissue biopsies for gene expression analysis where we found no significant change in IL-6 gene expression, suggesting that the increased serum IL-6 was not coming from adipose tissue. While we have not been able to determine the tissue origin of the increased serum IL-6 levels in these studies, the above evidence that muscle IL-6 increases insulin sensitivity[12, 13] whereas adipose tissue IL-6 does the opposite,[10] suggests that IL-6 may be coming from muscle in response to the HBOT and contributing to the HBOT-induced increased insulin sensitivity. Further

research into HBOT is recommended, including the use of techniques such as muscle biopsy to identify the source of IL-6 following HBOT. The role of soluble gp130 and the consequences of a decrease in serum levels following HBOT are not clear. However, the above discussion has indicated an important role for soluble gp130 to reduce the pro-inflammatory, "trans" signalling of IL-6. In fact, current research is exploring the potential for gp130 analogues to provide a clinical therapeutic benefit in their own right.[16, 17]

Our two studies found no change to cytokines other than gp130 and IL-6. Following HBOT given to men who were overweight or obese, there was no change to serum levels of TNF-α, MCP-1, HSP-70 or CRP. This was unexpected after our previous study where using a similar group of participants we found significant reductions in serum TNF-α and MCP-1 following HBOT.[4] While the small numbers within these studies was a limitation, the inconsistency of the cytokine response was disappointing and was not suggestive of a robust response. A review of the literature was undertaken for other studies that measured a cytokine response to HBOT. The discussion about IL-6 signalling has alluded to how intricately entwined the events surrounding cytokine action can be and I have excluded *in vitro* studies because of the isolated nature of their investigation. Neither have I considered animal studies but I have included results for some hormone responses as well as cytokines. The results are shown in Table 5. There is considerable variability in the reported results. Earlier studies encouragingly dismissed concern that the HBOT intervention may evoke a significant stress response reporting no change to cortisol and adrenaline.[18, 19] However, TNF-α has been reported to increase,[20] decrease,[4, 21] or remain unchanged.[22] No change is reported to serum IL-1β levels.[20-22] Relevant to the current studies, IL-6 has previously been reported to rise in response to HBOT in three publications[4, 22, 23] but others have found no change[20] or decreased IL-6 levels.[21]

Gene expression from adipose tissue biopsies showed little change after two HBOT interventions with only a decrease in MMP9 for the clamp technique study. MMP9 is a member of the matrix metalloproteinase family – enzymes that degrade basement membrane and extracellular matrix and are released under a variety of circumstances. In the acute response to injury they are important for tissue repair but their overexpression is also linked to activation of inflammatory pathways and pathological processes such as metastasis and cerebrovascular disease.[24] An earlier study performed adipose tissue

Author	Year	Participants	HBOT intervention	Outcomes
Tremellen[18]	1993	9 "healthy" participants	One HBOT to 2.0 or 2.8 atm abs, duration unknown. Blood 5 minutes after	No change to Adrenaline, Noradrenaline or Dopamine
Lund[19]	1999	8 male divers	One HBOT to 2.5 atm abs for 60 minutes. Blood 20 minutes after	↑ Endothelin-1, ↓ cortisol No change to Epinephrine, Norepinephrine, antidiuretic hormone, renin, aldosterone
Dennog[25]	1999	4 "healthy" participants	One HBOT to 2.5 atm abs for 60 minutes. Blood 24 hours later	↑ HSP-70 No change to vitamins A, C and E
Rocco[20]	2001	7 "healthy" participants	One HBOT to 2 atm abs and then 2.8 atm abs for an unknown duration. Blood 30 minutes after	↑ TNF-α, Endothelin-1 No change to IL-6, IL-1β
Schnittger[26]	2004	8 "healthy" participants	Three HBOT in 24 hours. 2.8 atm abs for 80 minutes. Blood before and after each HBOT	No change to IL-8, cortisol
Chen[27]	2006	31 patients with diabetes 29 "healthy" control	Daily HBOT for 3 days. 2.5 atm abs for 90 minutes. Blood after first and third HBOT	↓ insulin in control No change to growth hormone, insulin-like growth factor, leptin, IL-8
Alleva[22]	2008	20 patients with diabetes and non-healing wounds	Daily HBOT for 14 days. 2.5 atm abs for 75 minutes. Blood taken after 7 and 14 HBOT	↑ IL-6 No change to TNF-α, IL-1β
Wilkinson[4]	2015	11 men without diabetes 8 men with diabetes	Daily HBOT for 4 days. 2.0 atm abs for 90 minutes. Blood after 1 and 4 HBOT and 24 hours later	↓ TNF-α in all men ↓ MCP-1 and ↑ IL-6 in men without diabetes No change IL-1ra, IL-18
Bosco[21]	2018	23 men with avascular necrosis of femoral head	30 HBOT over 6 weeks, 30 day break then 30 more HBOT. Blood after 15 and 30 HBOT, and before and after second HBOT course	↓TNF-α, IL-6 at each time point No change to IL-1β
Anguiano-Hernandez[23]	2019	18 patients with diabetes and foot ulcers	20 HBOT over 4 weeks. 1.5 atm abs for 45 minutes. Bloods before and after course	↑ IL-6, adiponectin No change IL-10, interferon-γ

Table 5 (previous page): Published human studies investigating the effect of HBOT on cytokines and hormones.

biopsies on men who were overweight or obese, with and without diabetes, before and after four HBOT interventions.[4] In this study, we reported no change to gene expression for IL-6, MCP-1, TNF-α and IL-1ra. I found no other studies that investigated gene expression in human adipose tissue after HBOT. A study investigating HBOT in people with non-healing wounds measured gene expression from wound biopsy tissue.[22] They found an increase in MMP9 after 7 HBOT but a return to baseline after 14 HBOT. Other studies have measured the enzymatic activity of MMP9 in tissue. In rats using an ischaemic colonic anastomosis model, HBOT was associated with increased activity of MMP9 in the injured tissue and with improved outcome.[28] In a mouse model of impaired wound healing, HBOT was associated with upregulation of MMP9 activity in wound tissue and with improved healing.[29] Not surprisingly, MMP9 itself does not act in isolation. It interacts with other co-factors including the MMP9 inhibitor, TIMP1, which was unchanged in our studies. The small numbers are a limitation, however it is hard to make any conclusions from the gene expression data from our limited investigations.

Another limitation is that we have only investigated men for this insulin-sensitising effect of HBOT because of physiological variability in insulin sensitivity during the menstrual cycle in women, however we would expect similar responses in women.

In conclusion, while the literature displays considerable variability in the serum cytokine response to HBOT, we have found increased IL-6 and decreased soluble gp130 after HBOT in men who were overweight or obese. The glucose clamp study together with an earlier study[4] both found that the increase in IL-6 following HBOT was associated with an increase in insulin sensitivity using the glucose clamp technique. Further research should investigate the origin and the role of the increased IL-6 and its relationship with the insulin-sensitising effect of HBOT.

References

1. Heilbronn LK, Campbell LV. Adipose tissue macrophages, low grade inflammation and insulin resistance in human obesity. Curr Pharm Des 2008;14:1225-30
2. Hotamisligil GS. Inflammation and metabolic disorders. Nature 2006;444:860-7
3. Wilkinson D, Chapman IM, Heilbronn LK. Hyperbaric oxygen therapy improves peripheral insulin sensitivity in humans. Diabet Med 2012;29:986-9
4. Wilkinson D, Nolting M, Mahadi MK, Chapman I, Heilbronn L. Hyperbaric oxygen therapy increases insulin sensitivity in overweight men with and without type 2 diabetes. Diving Hyperb Med 2015;45:30-6
5. Wilkinson D, Szekely S, Gue B, Tam CS, Chapman I, Heilbronn LK. Assessment of Insulin Sensitivity during Hyperbaric Oxygen Therapy. Diving Hyperb Med;(in press)
6. Heilbronn LK, Rood J, Janderova L, Albu JB, Kelley DE, Ravussin E, et al. Relationship between serum resistin concentrations and insulin resistance in nonobese, obese, and obese diabetic subjects. J Clin Endocrinol Metab 2004;89:1844-8
7. Liu B, Hutchison AT, Thompson CH, Lange K, Heilbronn LK. Markers of adipose tissue inflammation are transiently elevated during intermittent fasting in women who are overweight or obese. Obes Res Clin Pract 2019;13:408-15
8. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr. Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest 2003;112:1796-808
9. Kern PA, Ranganathan S, Li C, Wood L, Ranganathan G. Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. American Journal of Physiology - Endocrinology & Metabolism 2001;280:E745-51
10. Bastard JP, Jardel C, Bruckert E, Blondy P, Capeau J, Laville M, et al. Elevated levels of interleukin 6 are reduced in serum and subcutaneous adipose tissue of obese women after weight loss. J Clin Endocrinol Metab 2000;85:3338-42
11. Carey AL, Steinberg GR, Macaulay SL, Thomas WG, Holmes AG, Ramm G, et al. Interleukin-6 increases insulin-stimulated glucose disposal in humans and

glucose uptake and fatty acid oxidation in vitro via AMP-activated protein kinase. Diabetes 2006;55:2688-97

12. Febbraio MA, Pedersen BK. Muscle-derived interleukin-6: mechanisms for activation and possible biological roles. FASEB J 2002;16:1335-47
13. Wojtaszewski JF, Jorgensen SB, Frosig C, MacDonald C, Birk JB, Richter EA. Insulin signalling: effects of prior exercise. Acta Physiol Scand 2003;178:321-8
14. Feve B, Bastard JP. The role of interleukins in insulin resistance and type 2 diabetes mellitus. Nature Reviews Endocrinology 2009;5:305-11
15. Cron L, Allen T, Febbraio MA. The role of gp130 receptor cytokines in the regulation of metabolic homeostasis. J Exp Biol 2016;219:259-65
16. Scheller J, Garbers C, Rose-John S. Interleukin-6: from basic biology to selective blockade of pro-inflammatory activities. Semin Immunol 2014;26:2-12
17. Morieri ML, Passaro A, Zuliani G. Interleukin-6 "Trans-Signaling" and Ischemic Vascular Disease: The Important Role of Soluble gp130. Mediators Inflamm 2017;2017 (no pagination)
18. Tremellen KP, Williamson JA, Frewin DB, Russell WJ. Plasma catecholamine levels during exposure to an environment of hyperbaric oxygen. Clin Auton Res 1993;3:91-3
19. Lund V, Kentala E, Scheinin H, Klossner J, Koskinen P, Jalonen J. Effect of hyperbaric conditions on plasma stress hormone levels and endothelin-1. Undersea Hyperb Med 1999;26:87-92
20. Rocco M, Antonelli M, Letizia V, Alampi D, Spadetta G, Passariello M, et al. Lipid peroxidation, circulating cytokine and endothelin 1 levels in healthy volunteers undergoing hyperbaric oxygenation. Minerva Anestesiol 2001;67:393-400
21. Bosco G, Vezzani G, Mrakic Sposta S, Rizzato A, Enten G, Abou-Samra A, et al. Hyperbaric oxygen therapy ameliorates osteonecrosis in patients by modulating inflammation and oxidative stress. J Enzyme Inhib Med Chem 2018;33:1501-5
22. Alleva R, Tomasetti M, Sartini D, Emanuelli M, Nasole E, Di Donato F, et al. alpha-Lipoic acid modulates extracellular matrix and angiogenesis gene expression in non-healing wounds treated with hyperbaric oxygen therapy. Mol Med 2008;14:175-83
23. Anguiano-Hernandez YM, Contreras-Mendez L, de Los Angeles Hernandez-Cueto M, Muand Oz-Medina JE, Santillan-Verde MA, Barbosa-Cabrera RE, et al.

Modification of HIF-1alpha, NF-akappaB, IGFBP-3, VEGF and adiponectin in diabetic foot ulcers treated with hyperbaric oxygen. Undersea Hyperb Med 2019;46:35-44

24. Cummins FJ, Jr., Gentene LJ. Hyperbaric oxygen effect on MMP-9 after a vascular insult. J Cardiovasc Transl Res 2010;3:683-7
25. Dennog C, Radermacher P, Barnett YA, Speit G. Antioxidant status in humans after exposure to hyperbaric oxygen. Mutat Res 1999;428:83-9
26. Schnittger V, Rosendahl K, Lind F, Palmblad J. Effects of carbon monoxide poisoning on neutrophil responses in patients treated with hyperbaric oxygen. J Investig Med 2004;52:523-30
27. Chen SJ, Yu CT, Cheng YL, Yu SY, Lo HC. Effects of hyperbaric oxygen therapy on circulating interleukin-8, nitric oxide, and insulin-like growth factors in patients with type 2 diabetes mellitus. Clin Biochem 2007;40:30-6
28. Azevedo LA, Parra RS, Da Rocha JJ, Ramalho LN, Ramalho FS, Feres O. Hyperbaric oxygen on the healing of ischemic colonic anastomosis--an experimental study in rats. Undersea Hyperb Med 2010;37:405-11
29. Sander AL, Henrich D, Muth CM, Marzi I, Barker JH, Frank JM. In vivo effect of hyperbaric oxygen on wound angiogenesis and epithelialization. Wound Repair Regen 2009;17:179-84

CHAPTER 6

6a. Introduction

The prospect of testing the insulin-sensitising effect of HBOT against hyperbaric air was envisaged early in the series of studies. However, basic questions related to onset and duration of this effect took precedence. The incremental experience gained in these interval studies better prepared us to return to the HBOT vs hyperbaric air question as a final study within this formal thesis. Study design could be kept simple as we had shown that the insulin-sensitising effect of HBOT was demonstrable during the first intervention. Although we had demonstrated the effect in men without diabetes, we wanted to make this study directly relevant to the issue of diabetes and so we recruited men with type 2 diabetes.

6b. Authorship statement

6c. Publication:

Wilkinson DC, Chapman IM, Heilbronn LK. Hyperbaric oxygen but not hyperbaric air increases insulin sensitivity in men with type 2 diabetes mellitus. Diving and Hyperbaric Medicine. (accepted for publication 22 August 2020)

Statement of Authorship

Title of Paper	Hyperbaric oxygen but not hyperbaric air increases insulin sensitivity in men with type 2 diabetes
Publication Status	☐ Published ☑ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Wilkinson DC, Chapman I, Heilbronn LK Diving and Hyperbaric Medicine Manuscript ID: DHM-2020-51/R1 RESUBMISSION - (680) Accepted for publication **22/08/2020**

Principal Author

Name of Principal Author (Candidate)	David Wilkinson		
Contribution to the Paper	Design study, acquisition and analysis of data, drafting manuscript, editing and approval of final version		
Overall percentage (%)	65 %		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	27/07/2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Ian Chapman		
Contribution to the Paper	Design study, acquisition and analysis of data, revising manuscript and approval of final version		
Signature		Date	27 JULY 2020

Name of Co-Author	Leonie Heilbronn		
Contribution to the Paper	Design study, acquisition and analysis of data, revising manuscript and approval of final version		
Signature		Date	27/7/2020.

Hyperbaric oxygen but not hyperbaric air increases insulin sensitivity in men with type 2 diabetes mellitus

David C. Wilkinson[1, 2]
Ian M. Chapman[2]
Leonie K. Heilbronn[2]

[1] Hyperbaric Medicine Unit, Royal Adelaide Hospital, Adelaide, Australia
[2] Adelaide Medical School, The University of Adelaide, Adelaide, Australia

Corresponding author:
Dr David Wilkinson
Hyperbaric Medicine Unit
Royal Adelaide Hospital
Port Road
Adelaide. SA 5000
Australia
Email: david.wilkinson@sa.gov.au

Keywords
Blood sugar level, Diabetes, Endocrinology, Hyperbaric research, Metabolism

Abstract

Introduction

We have previously shown that hyperbaric oxygen therapy (HBOT) increased insulin sensitivity in men who were obese or overweight, both with and without type 2 diabetes. The aim of this study was to test whether this insulin-sensitising effect is seen in hyperbaric air (HA).

Methods

Men with type 2 diabetes who were obese or overweight were randomised to 2 groups – HBOT (n = 13) or HA (n = 11). A hyperinsulinaemic euglycaemic glucose clamp (80 $mU.m^{-2}.min^{-1}$) was performed at baseline and during hyperbaric intervention. Both groups were compressed to 203 kPa (2 atmospheres absolute) for 90 minutes followed by a linear 30-minute decompression. The HBOT group breathed oxygen via a hood while the HA group breathed chamber air. Insulin sensitivity was assessed by the glucose infusion rate (GIR) during the last 30 minutes in the hyperbaric chamber (SS1) and the first 30 minutes after exit (SS2). Data were analysed for within-group effect by paired student t-test and between-group effect by one-way ANOVA.

Results

HBOT increased GIR by a mean 26% at SS1 (P = 0.04) and by 23% at SS2 (P = 0.018). There was no significant change in GIR during HA. A between-group effect was evident for the change in GIR at SS1 in HBOT vs HA (P = 0.036).

Conclusions

The pathway by which insulin sensitivity is increased in men with type 2 diabetes requires the high oxygen partial pressures of HBOT and should be further investigated. Insulin sensitivity was not changed in hyperbaric air.

Introduction

Hyperbaric Oxygen Therapy (HBOT) is defined as breathing near 100% oxygen while in a hyperbaric chamber pressurised to more than 101 kPa or 1 atmosphere absolute (atm abs).[1] HBOT administered by clinical facilities typically uses pressure between 203-284 kPa (2-2.8 atm abs), with a duration of treatment 90-120 minutes. HBOT is an evidence-based treatment for conditions including decompression illness, cerebral arterial gas embolism, necrotising fasciitis, non-healing ulcers and wounds and delayed radiation injuries.[1]

Although HBOT is not used to treat diabetes mellitus *per se*, the increasing prevalence of this disease means that diabetes, particularly type 2 diabetes, is a frequent co-morbidity in patients treated with HBOT. For some years, it has been apparent that people with diabetes who undergo HBOT may experience a decrease in their plasma glucose (PGL) during their treatment. Trytko *et al* used a hand-held glucometer to measure PGL before and after HBOT sessions in 27 patients with a mixture of type 1 and type 2 diabetes.[2] Over the 237 HBOT sessions they found a mean fall in PGL of 2.04 $mmol.L^{-1}$. Ekanayake *et al* measured laboratory glucose in a group of 5 patients with type 2 diabetes over the 2-hour duration of their HBOT session and found a mean fall of 3.5 $mmol.L^{-1}$ at the end of HBOT.[3] They found no change in serum insulin levels.

We investigated the effect of HBOT on insulin resistance and its reciprocal term, insulin sensitivity. Insulin resistance is defined as a relative impairment in the ability of insulin to exert its effect on glucose in target tissues (particularly muscle and liver). The development of insulin resistance is the best predictor for those likely to develop type 2 diabetes in the future.[4] Of the many investigative techniques used to assess insulin sensitivity, the hyperinsulinaemic euglycaemic glucose clamp is considered the gold standard.[5, 6] In recent studies we have described an acute effect of HBOT to increase insulin sensitivity, as measured with the glucose clamp technique. A pilot study initially revealed that insulin sensitivity was increased in a cohort of men with and without diabetes receiving a clinical course of HBOT.[7] Progressively we have demonstrated that insulin sensitivity was increased during the third HBOT session in a cohort of men with and without diabetes,[8] and recently that the increase can be measured during the first HBOT session.[9]

The aim of this study was to determine whether the insulin-sensitising effect seen during HBOT (while breathing oxygen at a very high partial pressure) is also present during an equivalent pressure excursion but using air as the breathing gas rather than oxygen.

Methods

The study was approved by the Human Research Ethics Committee of the Royal Adelaide Hospital (R20160801) and the University of Adelaide and entered on a trial registry site (NCT03138746, clinicaltrials.gov). The study was carried out in accordance with the Declaration of Helsinki. All participants provided written, informed consent. The study

was performed in the Hyperbaric Medicine Unit at the Royal Adelaide Hospital. Participant recruitment commenced in August 2018 and was closed due to reasons external to the study in December 2019.

PARTICIPANTS

Twenty-five participants were enrolled via a web-based recruitment company. Inclusion criteria were men aged 40 years or older who were obese or overweight (Body Mass Index (BMI) > 25 $kg.m^{-2}$) with type 2 diabetes. Exclusion criteria included the presence of significant other medical issues, other non-prescribed medication that could affect glucose homeostasis, smoking, individuals who regularly perform high intensity exercise (> twice per week) and current intake of > 140 g alcohol per week. All participants were assessed for fitness to enter the hyperbaric chamber by a hyperbaric physician (D.W.). Participants were randomised into two groups, HBOT and hyperbaric air (HA), stratified for BMI (BMI < 33 or BMI ≥ 33) by computer-generated, randomised block design in groups of 4.

STUDY DESIGN

Participants attended the Hyperbaric Medicine Unit on two occasions after overnight fasting (10 hours) and modification of their diabetic medication. On the first visit, participants sat in comfortable reclining chairs, breathing room air while the baseline glucose clamp was performed over 3½ hours. Intravenous cannulae were inserted, one in each forearm with one for the insulin and glucose infusions and the other for blood sampling. A primed insulin (Actrapid, Novo Nordisk, Baulkham Hills, Australia) solution (80 $mU.m^{-2}.min^{-1}$) was infused with blood samples taken at 5-10 minute intervals and PGL measured by a hand-held glucometer (Accu-Chek Performa, Roche Diagnostics, Sydney, Australia). PGL was clamped at 5.5 $mmol.L^{-1}$ with a variable infusion of 25% Dextrose (Baxter Healthcare, Old Toongabbie, Australia). Insulin sensitivity can be assessed at a pre-determined point in the glucose clamp during a steady state (SS) period when glucose infusion rate (GIR) and PGL readings are stable. We assessed insulin sensitivity by the GIR during two separate but consecutive 30-minute SS periods in the last hour of the infusion; SS1 corresponded with 2½-3 hours and SS2 with 3-3½ hours. The raw GIR data for each participant were adjusted for body surface area.

Two days later, participants returned after overnight fasting for a second glucose clamp using the same protocol, this time overlaid with a 2-hour session in the hyperbaric chamber. The insulin infusion was established one hour prior to entering the chamber. The large, triple-lock, multiplace hyperbaric chamber (Fink Engineering Pty Ltd, Warana, Australia) was compressed using air to 203 kPa (2 atm abs) and held at this pressure for 90 minutes followed by a 30-minute linear decompression back to ambient pressure. In the hyperbaric chamber, oxygen was delivered to the HBOT group via a hood system the same as used in clinical HBOT treatments (Amron International Inc, Vista, CA) which was connected on reaching 203 kPa pressure and continued for the 2-hour session (apart from a routine 5-minute "air-break" taken half-way through by temporarily detaching the hood). The HA group, who underwent the same pressure profile, breathed chamber air throughout the hyperbaric session. The participants remained in their reclining chairs once the clamp procedure had commenced and were wheeled into and out of the hyperbaric chamber. Blood samples were sent out of the chamber via the medical lock for PGL estimation. Insulin sensitivity was determined by the GIR during the same two SS periods, so SS1 coincided with the last 30 minutes of the 2-hour hyperbaric session and SS2 with the first 30 minutes after exit from the chamber. At each visit, blood was taken for serum insulin concentration before commencing the clamp infusions for fasting levels and during SS1 and SS2 to demonstrate hyperinsulinemia. Steady state insulin concentrations were not different between groups or between SS1 and SS2.

STATISTICAL CONSIDERATIONS

Statistical analyses were performed using Statistica (version 12, Statsoft, Tulsa, OK). Power analysis of earlier data suggested sample size of 20 in each group for power of 80% and α of 0.05. GIR data were normally distributed by Shapiro-Wilk and Kolmogorov-Smirnov tests. HBOT and HA groups were analysed by paired student t-test for within-group effects and ANOVA for between-group effect. Significance was considered at $P < 0.05$.

Results

Of the 25 men enrolled, one participant experienced technical issues during his hyperbaric session and loss of data required exclusion. The other 24 participants completed the study without complication and their characteristics are shown in Table 1.

	HBOT mean (SD)	HA mean (SD)
Age	62.3 (8.7)	56.3 (7.1)
Weight (kg)	108.2 (21.5)	102.4 (13.1)
Height (cm)	176.4 (6.7)	179.8 (10.3)
BMI ($kg.m^{-2}$)	34.7 (6.8)	31.8 (4.7)
BSA (m^2)	2.23 (0.21)	2.21 (0.17)

Table 1: Participant characteristics for HBOT (*n*=13) and hyperbaric air (*n*=11) groups. (BMI, body mass index; BSA, body surface area)

Medication	Number (*n* = 24)
Metformin	21
Insulin	5
SGLT-2 inhibitors	8
DPP-4 inhibitors	7
GLP-1 receptor agonists	4
Sulphonylureas	2

Table 2: Diabetes medication used by participants.

There were no significant differences between the two groups. <Insert Table 1> The participants were prescribed between one and four medications in the management of their diabetes (median two), summarised in Table 2. <Insert Table 2>

The GIR data for both the HBOT and HA groups at baseline and during the hyperbaric exposure, for SS1 and SS2 can be seen in Table 3. <Insert Table 3> Within the HBOT group, there was a mean 26% increase in GIR (median 17%) when compared to baseline, during SS1 ($P = 0.04$). There was a mean 23% increase in GIR (median 19%) during SS2 ($P = 0.018$). The HA group revealed no significant changes in GIR at SS1 or SS2.

One-way ANOVA for the change in GIR revealed a difference between groups at SS1 for HBOT vs HA (Figure 1, $P = 0.036$). A trend towards a between-group difference was evident at SS2 ($P = 0.088$). <Insert Figure 1>

	HBOT		Hyperbaric air	
	Baseline mean (SD)	Hyperbaric mean (SD)	Baseline mean (SD)	Hyperbaric mean (SD)
Steady State 1	151 (71)	177 (86)	180 (73)	166 (83)
Steady State 2	173 (87)	198 (85)	189 (91)	189 (81)

Table 3: Glucose infusion rates ($mg.m^{-2}.min^{-1}$) for HBOT and HA groups at baseline and during the hyperbaric intervention.

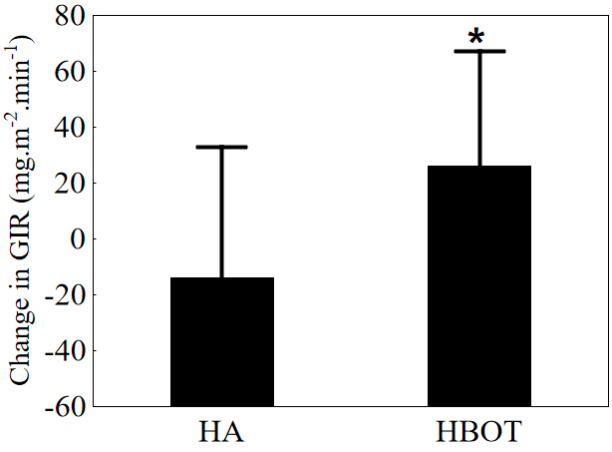


Figure 1: Change in Glucose Infusion Rate ($mg.m^{-2}.min^{-1}$), expressed as mean change and SD, for HA and HBOT groups at steady state 1. (*P = 0.036)

Discussion

This study has demonstrated that one session of HBOT significantly increased peripheral insulin sensitivity in men with type 2 diabetes, but exposure to an equivalent pressure profile without breathing supplemental oxygen (the hyperbaric air group) had no effect. The effect of HBOT persisted for at least the first 30 minutes after exit from the hyperbaric chamber.

The insulin-sensitising effect of HBOT observed in this study is consistent with that observed in our earlier studies. In a group of patients referred for clinical HBOT (5 men who were not obese and without diabetes and 5 men who were obese with type 2 diabetes), the glucose clamp revealed a significant increase in insulin sensitivity in the whole group during the third HBOT (37% increase) and the thirtieth HBOT (41% increase) although subgroup analysis revealed the change was statistically significant only in the group with diabetes.[7] A subsequent study recruited a cohort of men who were obese or overweight, both with (n = 8) and without (n = 11) type 2 diabetes.[8] A hyperinsulinemic euglycemic glucose clamp performed during the third HBOT demonstrated an increase in insulin sensitivity of 57% in those with type 2 diabetes and 29% in those without. We also found that the increase was still apparent during the first 30 minutes after exit from the hyperbaric chamber. A further study performed the glucose clamp technique during the first HBOT session on men who were obese or overweight but without diabetes (n = 9).[9] This demonstrated a significant 23% increase in insulin sensitivity during the first HBOT session. Encouragingly, the magnitude of the insulin-sensitising effect in the current study is comparable with the effect sizes previously published and is large enough to be clinically significant. The effect has an onset of action within one HBOT session but its duration is not known. However, this study again found

that the insulin-sensitising effect of HBOT was still active for at least the first 30 minutes after exit from the hyperbaric chamber.

The mechanism of action for the insulin sensitising effect of HBOT is also unknown. However, an important new contribution from this study is the finding that the hyperbaric air group showed no change in insulin sensitivity. We can say for the first time that the hyperbaric environment itself – where the increase in absolute pressure is transmitted throughout the human body and generates a number of recognised physiological responses – has no independent effect on insulin sensitivity, in men with diabetes at least; it also requires the very high oxygen partial pressures that are only delivered during clinical HBOT to increase insulin sensitivity. There have been no reports or studies that we are aware of to suggest that breathing high concentrations of oxygen *in the absence of hyperbaric conditions* affects insulin sensitivity, and it seems likely that both high oxygen concentrations and high pressures are needed to produce this effect.

Previous findings that this effect can be detected in men with and without diabetes suggest that HBOT initiates a common metabolic response which is not confined to people with diabetes mellitus.[8] If the underlying mechanism for this insulin-sensitising effect can be identified, it may offer a new therapeutic target. In earlier work we found the insulin-sensitising effect of HBOT was associated with some reductions in serum inflammatory cytokines,[8] however this may only be part of the story. A number of the therapeutic benefits of clinical HBOT have now been shown to require the deliberate generation of oxidative stress as a consequence of breathing hyperbaric oxygen.[10] Reactive oxygen species can be damaging to biological tissue, however they have other vital roles where they act as signalling molecules in a number of cellular pathways for a range of growth factors, cytokines and hormones.[11] Independently, other research has pointed out that reactive oxygen species can have both an inhibitory as well as a stimulatory effect on the intracellular glucose transport pathway.[12]

The finding that there was no change to insulin sensitivity in hyperbaric air is an important outcome in its own right. While this study was not specifically designed to answer SCUBA diving questions, it is interesting to consider that the hyperbaric air group undertook a simulated (dry) SCUBA dive, albeit perhaps not a typical dive profile. Their intervention was the equivalent of diving, on air, to 10 metres seawater for a 90-minute

bottom time followed by a very slow ascent to the surface over 30 minutes (so results for any "deeper" intervention cannot be assumed). This is relevant because people with diabetes do present to dive physicians with a desire to undertake SCUBA diving as recreation, with medical approval. For the dive physician, the medical assessment is complex and must consider the potentially disastrous consequences that could result from hypoglycaemia occurring underwater. Prospective observational studies have followed recreational divers with diabetes using detailed protocols for PGL management, suggesting that they can safely monitor and manage their PGL to allow diving.[13-15] However, it has never been determined if the potentially hazardous event encountered in hyperbaric medicine – the precipitous fall in PGL in a person with diabetes during HBOT – could also occur in response to the hyperbaric stimulus of the underwater environment. While other medical concerns will certainly exist for the potential diver with diabetes, this study provides the first evidence that exposure to a hyperbaric profile similar to that encountered in the recreational diving environment has no effect on insulin sensitivity. This encouraging finding may also be relevant to people in other hyperbaric environments.

One limitation to these studies is that we have only investigated men. Insulin sensitivity can change physiologically in adolescence and during pregnancy and different parts of the menstrual cycle in women. However, our studies have demonstrated an insulin-sensitising effect of HBOT that is not limited to those with diabetes and is likely to be a metabolic response to HBOT. As such, we would expect to see the same effect in women although this has never been tested. Other limitations include the relatively small sample size. Despite this, the magnitude of the effect is large enough to achieve statistical significance and is comparable to previous studies. The already labour-intensive glucose clamp was made more complicated by performing it within a hyperbaric chamber. Our previous studies have allowed us to develop experience in the use of this technique in the hyperbaric environment. Strategies include keeping participants sedentary in reclining chairs and wheeling them, plus the infusions, into and out of the hyperbaric chamber to minimise exertion. The regular blood samples were passed out of the hyperbaric chamber through the medical lock for PGL analysis while the glucometer itself utilised a glucose dehydrogenase reagent which is less affected by high oxygen environments.[16]

Conclusions

This study has further strengthened the evidence that acute exposure to hyperbaric oxygen leads to a clinically significant increase in insulin sensitivity in men with type 2 diabetes. This effect is still evident during the first 30 minutes after exit from the hyperbaric chamber although its duration beyond that time is not known. Importantly, we have shown for the first time that this insulin-sensitising effect does not occur when breathing hyperbaric air at 2 atm abs (10 msw equivalent). This may be relevant to other hyperbaric environments such as recreational diving but further work would be required to definitively establish the absence of an effect when breathing air at greater depths. The insulin-sensitising effect requires the very high partial pressures of oxygen only encountered during clinical HBOT. Further research should be encouraged to discover the mechanism for this novel effect on metabolism, as it could translate to new clinical therapies to improve glucose regulation.

Acknowledgements

The authors thank Dr. Bo Liu and the staff of the Hyperbaric Medicine Unit, Royal Adelaide Hospital for their help in performing this study.

I (D.W.) acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

Funding

This study was funded by the Royal Adelaide Hospital Research Committee.

Conflicts of Interest

None declared.

References

1. Weaver LK, editor. Hyperbaric Oxygen Therapy Indications. 13th ed. North Palm Beach, FL: Best Publishing Company; 2014.
2. Trytko B, Bennett MH. Blood sugar changes in diabetic patients undergoing hyperbaric oxygen therapy. SPUMS J. 2003;33(2):62-9.
3. Ekanayake L, Doolette DJ. Effects of hyperbaric oxygen treatment on blood sugar levels and insulin levels in diabetics. SPUMS J. 2001;31(1):16-20.

4. Warram JH, Martin BC, Krolewski AS, Soeldner JS, Kahn CR. Slow glucose removal rate and hyperinsulinemia precede the development of type II diabetes in the offspring of diabetic parents. Ann Intern Med. 1990;113(12):909-15. doi: 10.7326/0003-4819-113-12-909. PubMed PMID: 2240915.
5. Muniyappa R, Lee S, Chen H, Quon MJ. Current approaches for assessing insulin sensitivity and resistance in vivo: Advantages, limitations, and appropriate usage. Am J Physiol Endocrinol Metab. 2008;294(1):E15-E26. doi: 10.1152/ajpendo.00645.2007. PubMed PMID: 17957034.
6. Wallace TM, Matthews DR. The assessment of insulin resistance in man. Diabet Med. 2002;19(7):527-34. Doi: 10.1046/j.1464-5491.2002.00745.x. PubMed PMID: 12099954.
7. Wilkinson D, Chapman IM, Heilbronn LK. Hyperbaric oxygen therapy improves peripheral insulin sensitivity in humans. Diabet Med. 2012;29(8):986-9. doi: 10.1111/j.1464-5491.2012.03587.x. PubMed PMID: 22269009.
8. Wilkinson D, Nolting M, Mahadi MK, Chapman I, Heilbronn L. Hyperbaric oxygen therapy increases insulin sensitivity in overweight men with and without type 2 diabetes. Diving Hyperb Med. 2015;45(1):30-6. PubMed PMID: 25964036.
9. Wilkinson D, Szekely S, Gue B, Tam CS, Chapman I, Heilbronn LK. Assessment of Insulin Sensitivity during Hyperbaric Oxygen Therapy. Diving Hyperb Med.(in press).
10. Fosen KM, Thom SR. Hyperbaric oxygen, vasculogenic stem cells, and wound healing. Antioxid Redox Signal. 2014;21(11):1634-47. doi: 10.1089/ars.2014.5940. PubMed PMID: 24730726; PubMed Central PMCID: PMC4175035.
11. Thom SR. Hyperbaric oxygen: its mechanisms and efficacy. Plast Reconstr Surg. 2011;127 Suppl 1:131S-41S. doi: 10.1097/PRS.0b013e3181fbe2bf. PubMed PMID: 21200283; PubMed Central PMCID: PMC3058327.
12. Tiganis T. Reactive oxygen species and insulin resistance: the good, the bad and the ugly. Trends Pharmacol Sci. 2011;32(2):82-9. doi: 10.1016/j.tips.2010.11.006. PubMed PMID: 21159388.
13. Dear GDL, Pollock NW, Uguccioni DM, Dovenbarger J, Feinglos MN, Moon RE. Plasma glucose responses in recreational divers with insulin-requiring diabetes. Undersea Hyperb Med. 2004;31(3):291-301. PubMed PMID: 15568417.

14. Edge CJ, St Leger Dowse M, Bryson P. Scuba diving with diabetes mellitus--the UK experience 1991-2001. Undersea Hyperb Med. 2005;32(1):27-37. PubMed PMID: 15796312.
15. Pollock NW, Uguccioni DM, Dear GDL, Bates S, Albushies TM, Prosterman SA. Plasma glucose response to recreational diving in novice teenage divers with insulin-requiring diabetes mellitus. Undersea Hyperb Med. 2006;33(2):125-33. PubMed PMID: 16716063.
16. Tang Z, Louie RF, Lee JH, Lee DM, Miller EE, Kost GJ. Oxygen effects on glucose meter measurements with glucose dehydrogenase- and oxidase-based test strips for point-of-care testing. Crit Care Med. 2001;29(5):1062-70. doi: 10.1097/00003246-200105000-00038. PubMed PMID: 11378622.

CONCLUSIONS AND RECOMMENDATIONS

The hyperinsulinaemic euglycaemic glucose clamp has repeatedly identified a significant increase in peripheral insulin sensitivity during hyperbaric oxygen therapy (HBOT).[1-4] According to our studies, this effect comes on during the first HBOT intervention[3] and persists for at least the first 30 minutes after HBOT[2] although the duration is not known. The effect can be demonstrated in men without diabetes[2, 3] which suggests that HBOT is activating physiological pathways and is not specific to people with diabetes. As such, further research is encouraged as identification of this pathway may lead to new therapeutic targets to improve glucose regulation. The glucose clamp is the gold standard for assessing insulin sensitivity however it was disappointing that the frequently sampled intravenous glucose tolerance test (FSIGT) technique failed to find any changes.[3] In hindsight, there were a number of problems with the FSIGT and how it was used in the study and these issues may warrant revisiting, but it would be encouraging to demonstrate the insulin-sensitising effect of HBOT using alternative techniques of insulin sensitivity assessment. One technique to consider is the two-step glucose clamp using infusion of radioisotope-labelled glucose, which is a more complicated technique but would allow assessment of hepatic insulin sensitivity and the potential impact of an improvement in suppression of hepatic glucose production.

The final glucose clamp study showed there was no change to insulin sensitivity when breathing hyperbaric air.[4] The insulin-sensitising effect of HBOT requires both high inspired oxygen and increased pressure (2 atmospheres absolute), whereas breathing air at the equivalent of 10 metres sea water had no effect. This finding may be relevant to people in other hyperbaric environments such as diving. However, our simulated dive was relatively benign and further research is required to show that this effect is not activated by “deeper” diving profiles.

The chronic low grade inflammatory state associated with the development of insulin resistance, diabetes and other medical conditions has been shown to involve inflammatory cytokine release from adipose tissue.[5] Cytokines implicated in this pro-inflammatory process include TNF-α, MCP-1 and IL-6.[6] Our studies and a review of the literature suggest HBOT has an inconsistent effect on inflammatory cytokine release. In fact, two of our studies which showed an insulin-sensitising effect of HBOT also showed an

increase in serum IL-6 after HBOT.[2, 3] In one of these studies adipose tissue gene expression was measured and found no change in IL-6 expression in adipose tissue after HBOT.[2] The role of IL-6 has become far more complicated than simply a pro-inflammatory cytokine; consider the evidence that IL-6 is released from muscle during exercise,[7] which is an insulin-sensitiser.[8] It is possible that the increase in IL-6 after HBOT originates from skeletal muscle and this should be specifically investigated by techniques including muscle biopsy. However, changes to inflammatory cytokines may only be part of the story. Other pathways by which HBOT might influence insulin sensitivity and that deserve investigation involve oxidative stress mechanisms secondary to the high oxygen partial pressure. Enzymes within the intracellular insulin signalling pathway are very sensitive to reactive oxygen species with the effect to enhance insulin signalling.[9] It has already been demonstrated that HBOT stimulates the release of stem cells from bone marrow via reactive oxygen species mediated pathways.[10] The potential for HBOT to influence insulin sensitivity via oxidative stress mechanisms – the deliberated generation of reactive oxygen species – should be investigated by techniques including analysis of enzyme activity in fresh tissue samples.

Recommendations:

- The evidence supports continuing research into the insulin-sensitising effect of HBOT.
- The effect should be further defined by using alternative techniques for insulin sensitivity assessment, such as the two step glucose clamp with labelled glucose.
- Specific future study aims should include:
    - Determining the duration of the effect.
    - Performing the study in women.
- Dedicated “diving” studies testing more typical diving profiles should be undertaken to definitively show no change to insulin sensitivity in the diving environment.
- Specific investigation for the mechanism of the insulin-sensitising effect of HBOT should include the origin and the role of the increase in serum IL-6 after HBOT by techniques including muscle biopsy.

- Other specific investigations should consider HBOT-initiated oxidative stress mechanisms that influence enzymes within the intracellular insulin signalling pathway.

References

1. Wilkinson D, Chapman IM, Heilbronn LK. Hyperbaric oxygen therapy improves peripheral insulin sensitivity in humans. Diabet Med. 2012;29(8):986-9. doi: 10.1111/j.1464-5491.2012.03587.x. PubMed PMID: 22269009.
2. Wilkinson D, Nolting M, Mahadi MK, Chapman I, Heilbronn L. Hyperbaric oxygen therapy increases insulin sensitivity in overweight men with and without type 2 diabetes. Diving Hyperb Med. 2015;45(1):30-6. PubMed PMID: 25964036.
3. Wilkinson D, Szekely S, Gue B, Tam CS, Chapman I, Heilbronn LK. Assessment of Insulin Sensitivity during Hyperbaric Oxygen Therapy. Diving Hyperb Med. 2020;(in press).
4. Wilkinson DC, Chapman IM, Heilbronn LK. Hyperbaric oxygen but not hyperbaric air increases insulin sensitivity in men with type 2 diabetes mellitus. Diving Hyperb Med. 2020;(in press).
5. Hotamisligil GS. Inflammation and metabolic disorders. Nature. 2006;444(7121):860-7. doi: 10.1038/nature05485. PubMed PMID: 17167474.
6. Heilbronn LK, Campbell LV. Adipose tissue macrophages, low grade inflammation and insulin resistance in human obesity. Curr Pharm Des. 2008;14(12):1225-30. doi: 10.2174/138161208784246153. PubMed PMID: 18473870.
7. Febbraio MA, Pedersen BK. Muscle-derived interleukin-6: mechanisms for activation and possible biological roles. FASEB J. 2002;16(11):1335-47. doi: 10.1096/fj.01-0876rev. PubMed PMID: 12205025.
8. Wojtaszewski JF, Jorgensen SB, Frosig C, MacDonald C, Birk JB, Richter EA. Insulin signalling: effects of prior exercise. Acta Physiol Scand. 2003;178(4):321-8. doi: 10.1046/j.1365-201X.2003.01151.x. PubMed PMID: 12864736.
9. Tiganis T. Reactive oxygen species and insulin resistance: the good, the bad and the ugly. Trends Pharmacol Sci. 2011;32(2):82-9. doi: 10.1016/j.tips.2010.11.006. PubMed PMID: 21159388.

10. Thom SR, Bhopale VM, Velazquez OC, Goldstein LJ, Thom LH, Buerk DG. Stem cell mobilization by hyperbaric oxygen. American Journal of Physiology - Heart & Circulatory Physiology. 2006;290(4):H1378-86. doi: 10.1152/ajpheart.00888.2005. PubMed PMID: 16299259.