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Optimizing a method to analyse metabolic markers in leucocytes using Western Blot.

Version 2

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Abstract

Background: Elective surgery sets off a major stress reaction in the body in response to the invasive trauma. The surgical stress leads to a state of postoperative insulin resistance, resembling patients with type II diabetes. The insulin resistance is found to lead to an increased risk of infection due to inadequate activation leucocytes and the mechanisms behind it are still unknown.

Aim: The aim of this study was to optimize a method for isolating proteins from leucocytes, and to optimize the Western Blot analysis of the GLUT 1, GLUT 4, SNAT 1 and SNAT 2 proteins.

Materials and methods: Blood extracted from two individuals were used in five different protein isolation methods. The concentration of each isolate was measured followed by analysis using Western Blot in order to detect the presence of the specific proteins mentioned above.

Result: The data showed that there was no statistically significant difference between the protein isolation methods. Furthermore, GLUT 4 could not be analysed using Western Blot. In addition, the amount of protein sample analysed with Western Blot resulted in faint bands of the desired proteins.

Conclusion: Further optimization of the aforementioned methods is necessary in order for them to be useful within the research on leucocyte function during postoperative insulin resistance.

Keywords: Insulin resistance, Western Blot, optimization, leucocyte, GLUT 1, GLUT 4, SNAT 1, SNAT 2.

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Introduction

Elective surgery is the term for intentionally injuring the body in order to remove disease or restore damaged organs. As any invasive trauma to the body, this deliberate action sets off a series of reactions in the body, such as rapid neuroendocrine responses, the release of stress hormones and the triggering of cytokine and immune reactions [1]. This has great effects on the body and may result in numerous changes in the body metabolism, including protein breakdown, hypermetabolism and hyperglycaemia, putting the body in a general catabolic state [2,3]. In elective surgery, these metabolic alterations can be reversed by insulin [2]. However, the levels of insulin needed increase several-fold postoperatively, thus suggesting that the body is found to be in a temporary state of insulin resistance caused by the massive surgical stress.

Postoperative insulin resistance leads to the development of complications similar to those of diabetes, the only difference being that in surgery it takes days while in diabetes it can take up to weeks or months [4]. The state of postoperative insulin resistance causes an increase in glucose synthesis as well as a decrease in glucose uptake in the periphery, ultimately leading to hyperglycaemia [5]. In addition, glycogen formation is reduced due to the insulin resistance [6]. In skeletal muscle tissue it has been observed that the activation of glucose transporter (GLUT)-4 is reduced during insulin resistance [7]. This is probably linked to the reduced activity of insulin signalling system in the cells, for example PI-3 kinase [8]. Consequently these changes in glucose metabolism are very similar to those found in patients with type 2 diabetes, hence surgical patients with postoperative insulin resistance are prone to suffer similar complications as well [5].

It's important to point out that many of the complications due to insulin resistance involve cells that are not directly reliant on insulin for their glucose uptake. Leucocytes, endothelial cells and neurons are all cells that take up glucose independently of insulin; instead they depend on the existing glucose level in the plasma [5]. In addition these cells do not have the ability to store glucose, hence the only available pathway for the glucose to take is through glycolysis. Excess glucose in diabetic patients will overload the cells and eventually cause them to produce oxygen free radicals. The cells will take great damage and give rise to alterations in their gene expression, causing further inflammation in the body, which in turn will amplify the insulin resistance furthermore, creating a vicious cycle [9]. Studies from

muscle and adipose tissue have indicated that surgery triggers an increase in inflammatory signalling and that many of those signals are in accordance with those seen in diabetes, including overproduction of free radicals and activation of protein kinase C, promoting further inflammation [10,11].

A recent study in Montreal reported that the degree of insulin resistance is proportional to the risk of postoperative complications, especially serious infections [12]. Also there is a correlation between the size of the surgery and the degree of insulin resistance, thus suggesting that it has a negative impact in the activation of the immune system after surgery [13, 14]. It is well known that stress hormones, for example noradrenaline, glucocorticoids and glucagon are released after surgery and believed to play a role in the development of insulin resistance, yet studies show only slight elevations of these hormones and a weak correlation to the decrease in insulin sensitivity [15,16]. On the other hand, the concentrations of proinflammatory cytokines are prominently elevated after surgery and show strong correlation with the decline in postoperative insulin sensitivity [17,18]. This is also supported by the fact that several conditions involving chronic inflammatory diseases, like rheumatoid arthritis (RA), chronic kidney disease and obesity, all exhibit impaired insulin sensitivity [19-21]. Therefore it is not the stress hormones that play the biggest role in the development of peripheral insulin resistance, but the proinflammatory mediators that are the most responsible.

Activated leucocytes will demand an increased delivery of fuels in order to assist in immune responses [22]. In most cells, glucose is transported along a concentration gradient across the plasma membrane through sodium-independent facilitated diffusion with the help of transport proteins [23]. The transport proteins are coded and expressed by a family of glucose transporter genes (GLUT 1-5) with varying tissue specificity [22-24]. The two isoforms GLUT 1 and 4 have a molecular weight of 55 kDa and 50 kDa respectively [25,26]. Isoforms GLUT 1, 3 and 4 are known as high affinity transporters and operate almost at maximum rates [27]. This, in turn, means that their level of cell surface expression is an important factor for the rate of glucose uptake into the cells. In type 2 diabetic patients, the expression of glucose transporters in leucocytes decrease. This is proposed to contribute to the increased risk of infection [28]. Data on the relationship between postoperative insulin resistance and down regulation of glucose transporters in leucocytes is scarce in today's research.

Another metabolic marker in leucocytes that is of interest are the sodium-coupled amino acid transporters (SNATs). Amino acid transport system A is expressed generally in all tissues and is responsible for the uptake of small neutral amino acids through co-transport with sodium [28]. There are two isoforms, SNAT 1 and 2 that function through similar mechanisms in the cells [29]. SNAT 1 is coded by the SLC38A1 gene and has a molecular weight of 54 kDa. SNAT 2 is expressed through the SLC38A2 gene with a molecular weight of 56 kDa [30]. The transporter activity is strictly controlled by factors such as insulin, cortisol, oxygen availability and amino acid availability [31]. Further knowledge about intracellular signalling mechanisms is scarce and the effect of cytokines on the amino acid uptake very limited. However it was proposed that cytokines up-regulate the levels of amino acid transporters in pregnancies complicated by maternal diabetes and obesity [32]. There is still no information on how the expression of SNAT 1 and 2 are affected in leucocytes of patients suffering from postoperative insulin resistance.

The Western Blot technique is a method used to separate and distinguish specific proteins within a mixture of several proteins [33]. There are three steps to this technique that will ultimately lead to the ability to identify the desired proteins [34]. The first step is to separate proteins in a mixture from each other through gel electrophoresis. The proteins are divided based on molecular weight, and consequently by type. The second step is to transfer the separated proteins onto a membrane that acts as a solid support. The membrane is important for the last step, which is to mark the target proteins using an appropriate primary and secondary. Once marked with the respective antibodies, the target protein on the membrane can be visualised as bands. The thickness of the bands matches the amount of the protein, thus it provides a relative comparison of the present protein levels [34].

The knowledge about the role of GLUT and SNAT expression in leucocytes during insulin resistance is very limited today. The GLUT family is central to the regulation of glucose uptake; hence it is of interest to study their role in leucocyte function during postoperative insulin resistance. It would also be of interest to study whether SNAT family have any affect on leucocyte function since insulin is essential to their regulation.

Aim

The aim of this study was to optimize a method for analysing the expression of metabolic markers in leucocytes for future use within the research of postoperative insulin resistance.

Materials and methods

Ethics

Two individuals donated 6 ml of blood each for the sake of this study. The blood was taken from the blood bank at Örebro University Hospital, ensuring that both individuals were completely healthy and also completely anonymous to the research team. In addition, the Ethical Committee has certified this study to conduct this research.

Blood sampling and protein isolation from leucocytes

Blood from two healthy donors were collected in one EDTA vacutainer tube per donor.

The blood was handled directly upon retrieval and divided from one vacutainer tube into five test tubes containing 1 ml each. The erythrocytes in each sample were lysated with 1x Easy-Lyse (Dako, Glostrup, Denmark) for 15 minutes in dark. The lysated blood was then centrifuged at 600g for 10 minutes and the supernatant was then removed from the samples. The pellets consisting of leucocytes were washed with Phosphate-buffered saline (PBS) followed by centrifugation for 5 minutes at 300g and the removal of the resulting supernatant. A protease inhibitor (Sigma-Aldrich, St. Louis, MO, USA) was diluted 1:50 in cold Radioimmunoprecipitation (RIPA)-buffer (1% Igepal Ca 630 (Sigma-Aldrich), 0.5% Deoxycholic acid, 1% sodium dodecyl sulphate (SDS) and 97.5% PBS) and added to each pellet, which were then put in an ice bath. To isolate proteins from the leucocytes, five different methods were used. Sample 1 (S1) was left in the ice bath for 15 minutes and taken out for vortexing every other minute. Sample 2 (S2) was also left in the ice bath for 15 minutes, however it was drawn back and forth in a syringe every other minute. Samples 3-5 (S3-5) were ultra-sonicated (Diagenode Bioruptor) at the highest setting for 5, 10 and 15 minutes respectively. All the samples were then put in the centrifuge for 10 minutes at 10 000g at 4°C, the supernatants extracted into new tubes and the

formed pellets were discarded. The samples were stored at -80°C until further analysis.

Determining protein concentrations

The protein concentration was measured using the Bio-Rad DC™ Protein Assay Reagents Package (Hercules, CA, USA). Reagent S (Bio-Rad) was diluted 1:50 000 in Reagent A (Bio-Rad) to form solution A'. Protein standard (BSA, 10mg/ml) was diluted in RIPA-buffer (according to the following concentrations: 0 mg/ml, 0.2 mg/ml, 0.6 mg/ml, 1.0 mg/ml, 1.5 mg/ml and 2.0 mg/ml. The standards and samples were loaded in duplicates onto a microliter plate, thence 25 µl solution A' and 200 µl Reagent B (Bio-Rad) was added to each well. After 15 minutes of incubation in room temperature the samples were analysed using the Multiscan Ascent at an absorbance of 750 nm.

Western blotting

After determining the concentrations, 20 µg of protein sample were diluted 1:2 in 2x Laemmli sample buffer (Bio-Rad) and boiled at 100°C for 10 minutes in order to denature the proteins in the solution and prepare them for electrophoresis. The gel (7.5% Mini-PROTEAN® TGX™ Gel (Bio-Rad)) was loaded with 5 µl protein ladder (Precision Plus Protein™ Dual Colour (Bio-Rad)), followed by the protein samples, and run for 40 minutes at a setting of 200 V and 50 mA. Afterwards the gel was put in a bath of ice-cold transfer buffer (1x Tris-glycine and 0.005% SDS) for 10 minutes at 4°C. The separated proteins in the gel were transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad) through a wet transfer system at 100 V and 350 mA for one hour. Subsequently after completing the transfer, the membrane was washed in Tris buffered saline (TBS)-0.1% Tween-20 (TBST) for 20 minutes. The membrane was blocked for one hour with blocking buffer (2% ECL Advance Blocking Reagent (GE Healthcare, Fairfield, CT, USA) + 98% TBST) and washed in TBST. Subsequently, the membrane was incubated overnight with the indicated primary antibody, diluted to the desired concentration (see Table 1). The ß-actin (Abcam, Cambridge, England, United Kingdom) antibody was applied as an endogenous loading control for the membranes.

Table 1: Detailed information on the primary antibodies and the concentrations used.

Antibody	Company	Animal of origin	Clone	Antibody concentration
GLUT 1	Abcam	Mouse	SPM498	1:5000
GLUT 4	Cell Signalling technology	Mouse	1F8	1:1000-1:5000*
SNAT1	Abcam	Mouse	S104-32	1:1000
SNAT2	Abcam	Rabbit	Polyclonal	1:1000
β-actin	Abcam	Mouse	mAbcam 8226	1:10000

*The antibody was optimized twice and the concentration was changed from 1:1000 to 1:3000 and 1:5000 during the course of this study

After washing, the membrane was incubated with the secondary antibody goat anti mouse IgG HRP (1:50 000 (Abcam)) or goat anti rabbit IgG HRP (1:20 000 (Abcam)) diluted in blocking reagent. After incubation the membrane was washed in TBST. In a dark room, 5 ml Luminata^{TM} Forte Western HRP Substrate (Millipore, Billerica, MA, USA) was added to the membrane followed by incubation for 5 minutes. After the incubation the membrane was ready for analysis using Chemidoc XRS (Bio-Rad).

Membrane stripping

After the membrane was analysed it was stripped off its antibodies in order to apply the β-actin control antibody. The membrane was bathed in 1x stripping buffer (0.4% ß-mercapto-ethanol, 1% SDS, 3% 1M Tris pH 6.7 and 95.6% MQ H_2O) heated to 50°C for 30 minutes. After washing it in PBS and TBST, the membrane was ready to be used from the first blocking step.

Results

Protein concentrations in isolates

The data from the protein concentration measurement is presented in Table 2. Ultra sonication for 15 minutes (S6) yielded the highest concentration of protein in individual A, whereas ultra sonication for 10 minutes (S5) yielded the lowest concentration. The highest concentration of protein in individual B was obtained by S5, and the lowest was yielded when putting the sample in an ice bath and vortexing it every other minute (S1). Individual A presented with a higher concentration than individual B with every method.

Table 2. Concentration of total protein measured from single sample isolates in both individuals.

Method	Protein concentration (mg/ml)	
	Individual A	Individual B
S1	1.71	1.11
S2	1.71	1.19
S3	1.81	1.47
S4	1.59	1.58
S5	1.86	1.22

Protein detection using Western Blot

Method S2 yielded insufficient amounts sample in order to conduct further Western Blot analysis; hence it was excluded from this experiment.

Bands of GLUT 1 were detected within a range of 25-37 kDa (expected molecular weight: 55 kDa) at a dilution of 1:5000 (Fig.1). The GLUT 4 antibody concentration was optimized first from 1:1000 to 1:3000 and then from 1:3000 to 1:5000. However, no bands of GLUT 4 were seen on the membrane (data not shown). Both the SNAT1 (expected molecular weight: 56 kDa) (Fig. 2) and SNAT2 (expected molecular weight: 54 kDa) (Fig.3) proteins were detected at a molecular weight between 75 and 50 kDa. Both SNAT proteins are presented as faint bands.

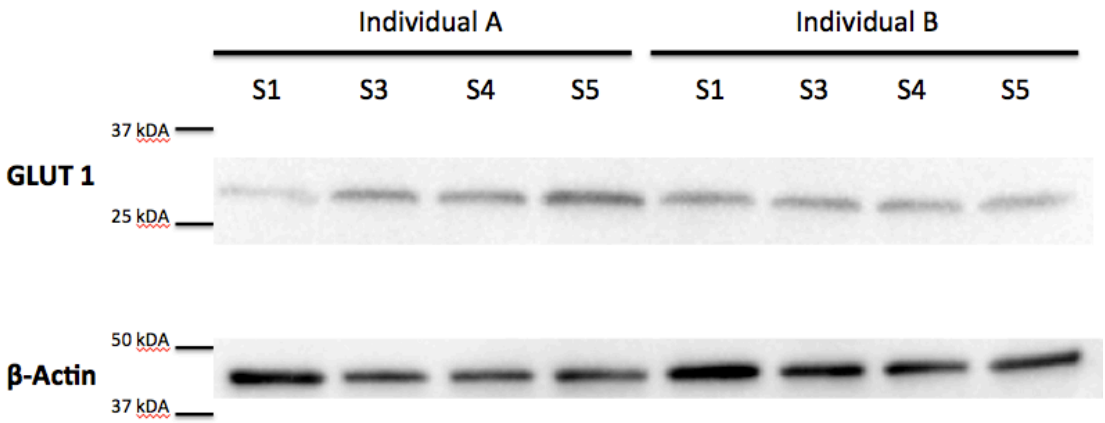

Figure 1. The expression of GLUT 1 (1:5000) using Western Blot with β-actin loading control. (1:20 000). Molecular weight measured in kDa.

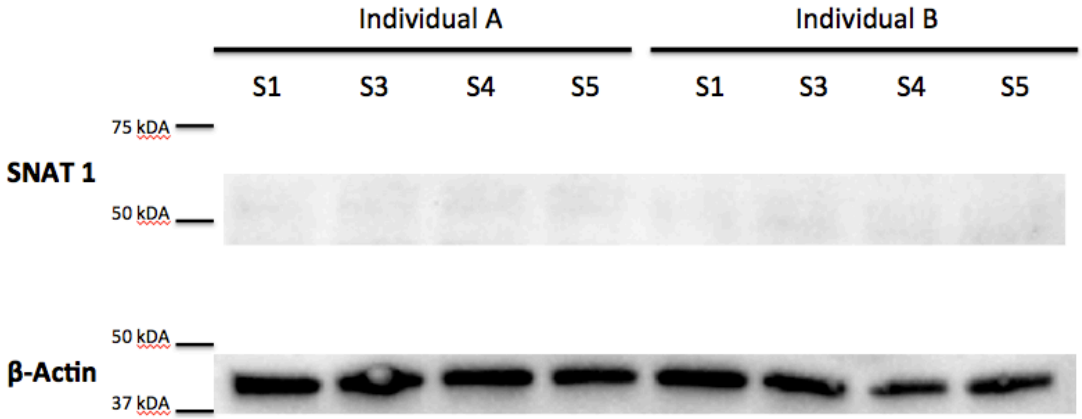

Figure 2. Detection of the SNAT1 (diluted 1:1000) using Western Blot with B-actin loading control. Molecular weight measured in kDa.

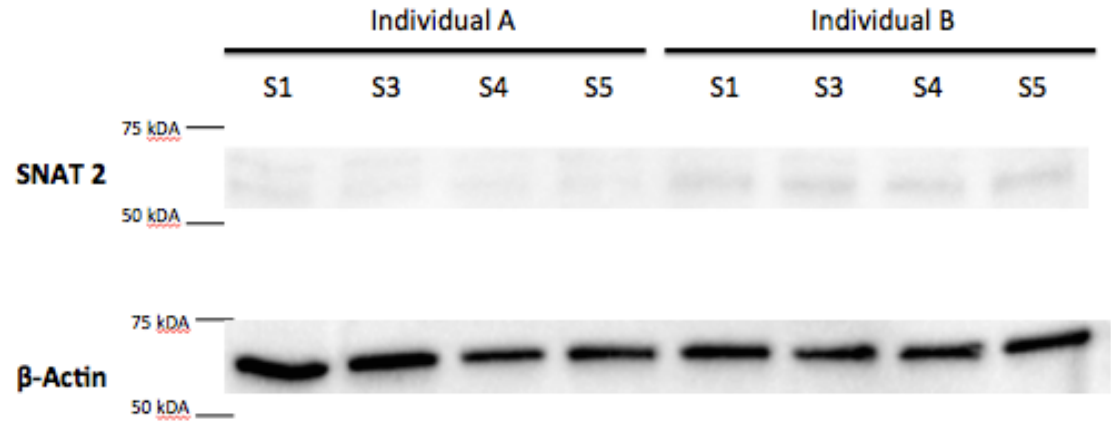

Figure 3. SNAT2 (diluted 1:3000) expression using Western Blot analysis with the B-actin loading control. Molecular weight measured in kDa.

Discussion

It is not possible from these results to determine which method of protein isolation was better. First of all the population studied was too small hence there is no statistical significance. Also the results were inconsistent both within a single individual and between both individuals. Method S4 yielded the highest concentration for individual B; yet, in individual A it yielded the lowest (Table 2). This study, however, indicates that method S2 was the least optimal since the amount of protein isolate retrieved was insufficient in both individuals and thus excluded from the

Western Blot analysis. Nevertheless, these results were too inconclusive; henceforth there was no difference between the different protein isolation methods.

There was a great difference in total protein concentration between the two individuals, which could not be disregarded (Table 2). Likely reasons for this could be many, for instance the individual variation within a population. The personal information about the donors was undisclosed, hence there was no information about their age, gender or ethnical background, all of which are important factors for leucocyte function. Also, the leucocyte count was not measured in the blood samples before the isolation; hence there is a possibility that the individuals had different normal levels of leucocytes. Since the population of this study was too small, the difference in protein concentration may have been coincidental.

The GLUT 1 bands were detected within the range of 25-37 kDa, which is considerably lower than the known molecular weight of 55 kDa. Furthermore the bands were consistently intense for all samples and there was no unspecific background binding of the antibody. This observation has not been made in any other study using the same antibody, however, Morato et al. found the bands within the appropriate range with a 1:1000 dilution of the antibody. Any other differences in protocol were not revealed [35]. A possible explanation to this result is that the bands observed are of another protein and the bands of GLUT 1 were too weak to be detected. Perhaps the antibody bound to a different protein, which had the same epitope as GLUT 1 and was available in a larger concentration in the isolates. It may otherwise have been a cross reaction between the antibody and a different epitope belonging to another protein. Other suggestions to improve the GLUT 1 analysis would be to increase the amount of protein loaded onto the electrophoresis gels. Consequently it would require a bigger blood sample from the donor in order to extract a larger quantity of protein isolate. So a recommendation when repeating this experiment would be to load the electrophoresis gels with 30 µg and increasing the amount to 40 µg if needed. This would not guarantee an adequate amount of proteins, however it is the first step towards optimizing this method even further. Another suggestion would be to change the antibody dilution to a higher concentration in order to let more antibodies bind to the correct protein.

The membrane loaded with the GLUT 4 was problematic, as the primary antibody concentration had to be changed twice, but the bands could still not be detected. The results of the β-actin control excluded any possibilities that there may

have been any errors during the blocking. Instead, the problem was most likely due to the GLUT 4 antibody. One possible explanation is that the primary antibody produced by the company was inadequate or faulty, and an antibody from a different company should be used in future studies. Another explanation could be that the antibody needed to be diluted even further because the concentrations used for this experiment were too strong and lead to unspecific bindings all over the membrane. It would be recommended to test the latter before switching to an antibody of another brand. Previous studies conducted by Gross et al. successfully found bands of GLUT 4, although they loaded their electrophoresis gel with 200 µg of protein from whole cell lysates. The antibody concentration was not apparent in the study [36]. The problems caused by repeating the GLUT 4 analysis several times was that it consumed too much of the protein isolate. In order to identify the source of error, the Western Blot analysis had to be done over. Even though the problem was most likely identified, the process was both time and material consuming.

Both SNAT proteins were detected within the expected size range, however the bands observed were very weak. This suggests either a low presence of SNAT 1 and 2 in the analysed membranes or an inadequate concentration of antibody leading to a weak binding to the proteins. Should it be the former reason, the first recommendation would be to increase the amount of protein loaded onto the gels during electrophoresis. So instead of 20 µg of protein per sample, the electrophoresis should be tested with 30 µg first and increased further to 40 µg if the results are unchanged. Alternatively, the SNAT antibodies were too weakly bound to its target proteins, presenting weak bands in the Western Blot analysis. In order to test this theory, it is recommended that the antibodies be diluted into a higher concentration. There are no previous references to the specific antibody used for SNAT 1; hence there is no evidence to support any of the theories behind the outcome of the results in this study. In contrast, Oh et al. retrieved similar results using the same antibody as in this study in the SNAT 2 analysis. The major difference in protocol that was evident was the use of a nitrocellulose membrane instead of a PVDF-membrane [37].

Western Blot analysis is a method involving many different steps of preparation. Consequently this means a lot of sources of errors that may be difficult to identify. For example the blocking of the membrane may be inadequate so that it’s not prepared to be loaded with the primary antibody, or there may be something wrong with the antibody concentration as observed in this study. Errors may also arise

during the electrophoresis or during the transfer phase. The biggest problem however, is the lack of a standardized protocol for Western Blot. The team at the Centre of Clinical Research at Örebro University Hospital developed the protocol used for this study and is not the same at other research facilities. There is no standard protocol used for all Western Blot studies, which reduces the quality of this method.
The results from this study were unsatisfactory and the reasons behind it were simply the lack of time, the small population size and the limited amount of material used. Analysis of one protein together with its control took in total four days. As the time given to conduct this study was very limited it lead to using a small population size. The improvements to be made if more time was given would be to use a bigger population in order to retrieve results of statistical significance. Also, bigger blood samples are recommended for further optimization. Bigger blood samples would generate increased amounts of protein isolates, which increases the amount of material to work with in the Western Blot analysis.

Conclusion

The results of this study show that there is no difference between the protein isolation methods and the extracted protein concentration. However, the choice and concentration of primary antibodies does have an affect on the quality of the Western Blot analysis. The material used in this study was insufficient, thus it is essential to further optimize these methods before application within the actual research on leucocyte function during postoperative insulin resistance.

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References

[1] Ljungqvist O, Fearon K, Little RA. Clinical nutrition. In: Gibney MJ, Elia M, Ljungqvist O, Dowsett J, editors. The Human Nutrition Textbook Series Oxford, UK: Blackwell Science; 2005.

[2] Brandi LS, Frediani M, Oleggini M, Mosca F, Cerri M, Boni C, et al. Insulin resistance after surgery: normalization by insulin treatment. *Clin Sci (Lond)* November 1990;79(5):443-50.

[3] Svanfeldt M, Thorell A, Nygren J, Ljungqvist O. Postoperative parenteral nutrition while proactively minimizing insulin resistance. *Nutrition* 2006;22(5):457 - 64.

[4] Ljungqvist O, Nygren J, Thorell A. Modulation of post-operative insulin resistance by pre-operative carbohydrate loading. *Proc Nutr Soc*. 2002;61(3):329-336.

[5] Ljungqvist O. Jonathan E. Rhoads Lecture 2011: Insulin Resistance and Enhanced Recovery After Surgery. *JPEN* July 2012;36(4):389-398.

[6] Ljungqvist O, Nygren J, Thorell A, Brodin U, Efendic S. Preoperative nutrition: elective surgery in the fed or the overnight fasted state. *Clin Nutr* 2001;20(1):167-171.

[7] Thorell A, Nygren J, Hirshman M, Hayashi T, Nair KS, Horton ES, et al. Surgery-induced insulin resistance in human patients: relation to glucose transport and utilization. *Am J Physiol* 1999;276(4):754-761.

[8] Wang Z, Wang Q, Wang W, Qin H. Randomized clinical trial to compare the effects of preoperative oral carbohydrate versus placebo on insulin resistance after colorectal surgery. *Br J Surg* 2010;97(3):317-327.

[9] Brownlee M. The pathobiology of diabetic complications: a unifying mechanism. 2005;54(6):1615-1625. *Diabetes* 2005;54(6):1615-1625.

[10] Witasp A, Nordfors L, Schalling M, Nygren J, Ljungqvist O, Thorell A. Increased expression of inflammatory pathway genes in skeletal muscle during surgery. *Clin Nutr.* 2009;28(3):291-298.

[11] Witasp A, Nordfors L, Schalling M, Nygren J, Ljungqvist O, Thorell A. Expression of inflammatory and insulin signaling genes in adipose tissue in response to elective surgery. *J Clin Endocrinol Metabol* 2010;95(7):3460-3469.

[12] Sato H, Carvalho G, Sato T, Lattermann R, Matsukawa T, Schricker T. The association of preoperative glycemic control, intraoperative insulin sensitivity, and outcomes after cardiac surgery. *J Clin Endocrinol Metabol* 2010;95(9):4338-4344.

[13] Soop M, Nygren J, Thorell A, Weidenhielm L, Lundberg M, Hammarqvist F, et al. Preoperative oral carbohydrate treatment attenuates endogenous glucose release 3 days after surgery. *Clin Nutr* 2004 Aug;23(4):733-741.

[14] Kahaodiar L, McCowen K, Bistrian B. Perioperative hyperglycemia, infection or risk? *Curr Opin Clin Nutr Metab Care* 1999;2(1):79-82.

[15] Thorell A, Loftenius A, Andersson B, Ljungqvist O.
Postoperative insulin resistance and circulating concentrations of stress hormones and cytokines. *Clin Nutr* 15:75–79. 1996 Apr;15(2):75-79.

[16] Thorell A, Efendic S, Gutniak M, Häggmark T, Ljungqvist O. Insulin resistance after abdominal surgery. *Br J Surg* 1994;81(1):59-63.

[17] Kremen J, Dolinkova M, Krajickova J, Blaha J, Anderlova K, Lacinova Z, et al. Increased subcutaneous and epicardial adipose tissue production of proinflammatory cytokines in cardiac surgery patients: possible role in postoperative insulin resistance. *J Clin Endocrinol Metabol 2006;91(11):4620-4627.*

[18] Gletsu N, Lin E, Zhu J, Khaitan L, Ramshaw B, Farmer P, et al. Increased plasma interleukin 6 concentrations and exaggerated adipose tissue interleukin 6 content in severely obese patients after operative trauma. *Surgery* 2006;140(1):50-57.

[19] Shinohara K, Shoji T, Emoto M, Tahara H, Koyama H, Ishimura E, et al. Insulin resistance as an independent predictor of cardiovascular mortality in patients with end-stage renal disease. *J Am Soc Nephrol* 2002;13(7):1894-1900.

[20]. Hotamisligil G, Arner P, Caro J, Atkinson R, Spiegelman B. Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. *J Clin Invest* 1995;95(5):2409-2415.

[21] Dessein P, Joffe B, Stanwix A. Inflammation, insulin resistance, and aberrant lipid metabolism as cardiovascular risk factors in rheumatoid arthritis. *J Rheumatol* 2003;30(7):1403-1405.

[22] Fu Y, Maianu L, Melbert B, Garvey W. Facilitative glucose transporter gene expression in human lymphocytes, monocytes, and macrophages: a role for GLUT isoforms 1, 3, and 5 in the immune response and foam cell formation. *Blood Cells Mol Dis* 2004;32(1):182-190.

[23] Korgun E, Demir R, Sedlmayr P, Desoye G, Arikan G, Puerstner P, et al. Sustained hypoglycemia affects glucose transporter expression of human blood leukocytes. *Blood Cells Mol Dis* 2002;28(2):152-159.

[24] Mueckler M. Facilitative glucose transporters. *Eur J Biochem* 1994;219(3):713-725.

[25] Yu S, Ding W. The 45 kDa form of glucose transporter 1 (GLUT1) is localized in oligodendrocyte and astrocyte but not in microglia in the rat brain. *Brain Res* 1998;797(1):65-72.

[26] Lizunov V, Stenkula K, Lisinski I, Gavrilova O, Yver D, Chadt A, et al. Insulin stimulates fusion, but not tethering, of GLUT4 vesicles in skeletal muscle of HA-GLUT4-GFP transgenic mice. *Am J Physiol Endocrinol Metabol* 2012;302(8):950-960.

[27] Kipmen-Korgun D, Bilmen-Sarikcioglu S, Altunbas H, Demir R, Korgun E. Type-2 diabetes down-regulates glucose transporter proteins and genes of the human blood leukocytes. *Scand J Clin Lab Invest* 2009;69(3):350-358.

[28] McGivan J, Pastor-Anglada M. Regulatory and molecular aspects of mammalian amino acid transport. *Biochem J* 1994;299(2):321-334.

[29 Hatanaka T, Huang W, Wang H, Sugawara M, Prasad P, Leibach F, et al. Primary structure, functional characteristics and tissue expression pattern of human ATA2, a subtype of amino acid transport system A. *Biochim Biophys Acta* 2000;1467(1):1-6.

[30] Alexander S, Benson H, Faccenda E, Pawson A, Sharman J, Spedding M, et al. The Concise Guide to PHARMACOLOGY 2013/14: Transporters. *Br J Pharmacol* 2013;170:1706-1796.

[31] Jones H, Powell T, Jansson T. Regulation of placental nutrient transport - a review. *Placenta* 2007;28(8-9):763-774.

[32] Jones H, Jansson T, Powell T. IL-6 stimulates system A amino acid transporter activity in trophoblast cells through STAT3 and increased expression of SNAT2. *Am J Physiol Cell Physiol* 2009;297(5):1228-1235.

[33] Dennis-Sykes CA, Miller WJ, McAleer WJ. A quantative Western Blot method for protein measurement. *J Biol Stand* 1985;13(4):309-14.

[34] Mahmood T, Tang PC. Western Blot: Technique, Theory, and Trouble Shooting. *N Am J Med Sci* 2012; 4(9):429-434.

[35] Morato P, Lollo P, Moura C, Batista T, Camargo R, Carneiro E, et al. Whey Protein Hydrolysate Increases Translocation of GLUT-4 to the Plasma Membrane Independent of Insulin in Wistar Rats. *PLoS ONE* 2013;8(8):25-05-14-e71134.

[36] Gross D, Farmer S, Pilch P. Glut4 Storage Vesicles without Glut4: Transcriptional Regulation of Insulin-Dependent Vesicular Traffic. *Mol Cell Biol* 2004;24(16):7151-7162.

[37] Oh R, Pan W, Yalcin A, Zhang H, Guilarte T, Hotamisligil G, et al. Functional RNA Interference (RNAi) Screen Identifies System A Neutral Amino Acid Transporter 2 (SNAT2) as a Mediator of Arsenic-induced Endoplasmic Reticulum Stress. *J Biol Chem* 2012;287(8):6025-6034