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Original article

Is the quickness of resuscitation after hypoxia influenced by the oxygen concentration? Metabolomics in piglets resuscitated with different oxygen concentrations

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Proceedings

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Abstract

Perinatal asphyxia is one of the leading causes of morbidity and mortality in the neonatal period. There is an on-going debate in the literature concerning the correct oxygen concentration to be used during neonatal resuscitation. Aim of this study was to investigate whether different metabolic profiles occurred according to oxygen concentration administered and quickness of resuscitation. We tested the hypothesis that the metabolic profile may be affected by the response to the different oxygen concentration and influenced the different time of recovery. Forty male Landrace/Large newborn piglets were the subjects of the present study. As a consequence of the different time of resuscitation, a metabolomics analysis between the two classes of reoxygenated piglets with the slowest and fastest recovery was carried out: first group (4 piglets) RT < 15 minutes and second group (6 piglets) RT > 68 minutes. In addition, ^{1}H-NMR metabolomics study was performed showing different metabolic profiles between the two groups. The most

significant metabolites were: N-phenylacetylglycine, acetoacetate, methanol, glucose, sarcosine, succinate, dimethylamine and alanine. Our results seem to indicate that the rapidity of resuscitation is influenced by the oxygen concentration.

Keywords

Metabolomics, asphyxia, resuscitation, oxygen, urine, newborn, piglets.

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Introduction

It has been described that perinatal asphyxia can cause injury to the nervous, cardiovascular and respiratory systems and among the affected newborns, important clinical disability are observed, but cannot be predicted. A debate in the literature exists concerning the correct oxygen concentration to be used during neonatal resuscitation, and in this regard, it has been suggested the need for a critical review [1-3]. In particular, studies clearly demonstrated that the use of low oxygen concentration causes less damage by free radicals molecules. In fact, it is known that the therapy with high oxygen concentration after an hypoxic insult generates a condition of oxidative stress and multiorgan damage [4] which is also closely related to the recovery time. Less recovery time will result in a lower extent of damage due to hypoxia condition [5]. As matter of the fact, perinatal asphyxia and reoxygenation can cause large changes in the urinary metabolome [6, 7]. Therefore, the metabolomic approach could be the key to find modifications in the metabolic pathways that are crucial for this condition.

Metabolomics applications and techniques are in an exponential growth phase and it is already clear that this strategy will have a significant impact on the discovery of new clinical information. The aim of this study is to find different metabolic profiles correlated to the time of recovery. We tested the hypothesis that metabolic profile may be affected by the response to different oxygen concentration as therapy.

Materials and methods

Forty male Landrace/Large newborn piglets, weighing 2.3-3.8 kg, were the subjects of the present study. Animals were sedated with an intramuscular injection of ketamine 10 mg/kg (Narketan, Vétoquinol UK Ltd) and midazolam 0.5 mg/kg (Dormicum®, Hoffmann-La Roche, Germany). Venous access was established via the marginal auricular vein and anesthesia was induced by administration of propofol 1 mg/kg (Diprivan, AstraZeneca) and fentanyl 10 μg/kg (Fentanyl, Janssen-Cilag). Animals were then intubated. Normal saline 0.9% 10 ml/kg/h and 5 ml/kg/h of dextrose in water 5% were infused to prevent dehydration and hypoglycemia. Heart rate (HR), electrocardiogram (ECG), saturation of oxygen by pulse oximeter (SpO_2) and rectal temperature (Matron, BPM 1000, VET, ET Medical Devices Spa) were monitored non-invasively. Body temperature was maintained at 38 ± 1°C with a table heating pad and an overhead-heating lamp. An intravenous bolus of fentanyl 20 μg/kg and cis-atracurium 0.2 mg/kg (Nimbex, Abbott) were administered, after which they were mechanically ventilated (Soxil, Soxitronic, Felino, Italy). Ventilatory settings: tidal volume 10-15 ml/kg, pressure 19 cm H_2O and respiratory rate 30-40 breaths/minute aiming at end-tidal CO_2 ($ETCO_2$) of 35-45 mm Hg. The fraction of inspired oxygen (FiO_2) was adjusted between 0.21 and 0.25 in order to maintain target SpO_2 90-95%. Infusion of 8-10 mg/kg/h propofol and boluses of 10 μg/kg fentanyl and 0.15 mg/kg cis-atracurium maintained anesthesia. The right internal jugular vein and carotid artery were catheterized, via a paratracheal incision, with single-lumen catheters (S1UVC5.0, NeoCare®; Klein-Baker Medical Co., San Antonio, TX, USA) which were connected to external transducers (Transpac, Abbott Critical Care Systems, USA), for continuous monitoring of central venous pressure, systolic, mean and diastolic pressure of the carotid artery. The animals were stabilized for 30 minutes prior to experimentation. The inspired FiO_2 was then decreased to 0.06-0.08 to induce hypoxia, while the animals were maintained on the same settings of ventilation. Monitoring aimed at detecting either bradycardia (HR < 60 beats per minute) or severe hypotension (MAP < 15 mm Hg) was performed. As soon as hemodynamic compromise occurred, hypoxemia (pO_2 30-50 mm Hg) was confirmed

on arterial blood gases and resuscitation began according to the Newborn Life Support (NLS) algorithm [6]. Animals were distributed in 4 groups (10 animals for each group) and were resuscitated with 4 different O_2 concentration: 18%, 21%, 40% and 100% respectively, until HR and MAP returned to 90% of baseline levels.

Sample collection

Urine samples were collected from each animal at different time points: a baseline sample was obtained before to start the hypoxia process and a second urine sample once the animals were reoxygenated (**Fig. 1**). Urine sample was centrifuged to remove insoluble material and, in order to avoid the growth of bacteria, 10 µl of sodium azide solution (NaN_3 in H_20) was added and stored at -80°C. Urine were prepared according to the ^{1}H-NMR protocol after being thawed and centrifuged at 12,000 x rpm for 10 min at 4°C. 400 µL of urine were added to a solution of 200 µL of phosphate buffer solution pH 7.4 and 50 µL of TSP (trimethylsilyl propanoic acid) in D_20 10 mM (f.c. 1 mM). Then the mixture was dispensed into a 5 mm NMR tube (Newera, USA).

^{1}H-NMR experiments

All ^{1}H-NMR spectra were carried out on a Varian UNITY INOVA 400 spectrometer. All samples were submitted to identical standard acquisition parameters and pulse. The sequence used was TNNOESY with mixing time of 0.150 seconds, a sat-delay of 2 s and a sat-power of 2. Spectra were recorded at 300 K with a spectral width of 6,000 Hz, a 90° pulse, an acquisition time of 2 s, a relaxation

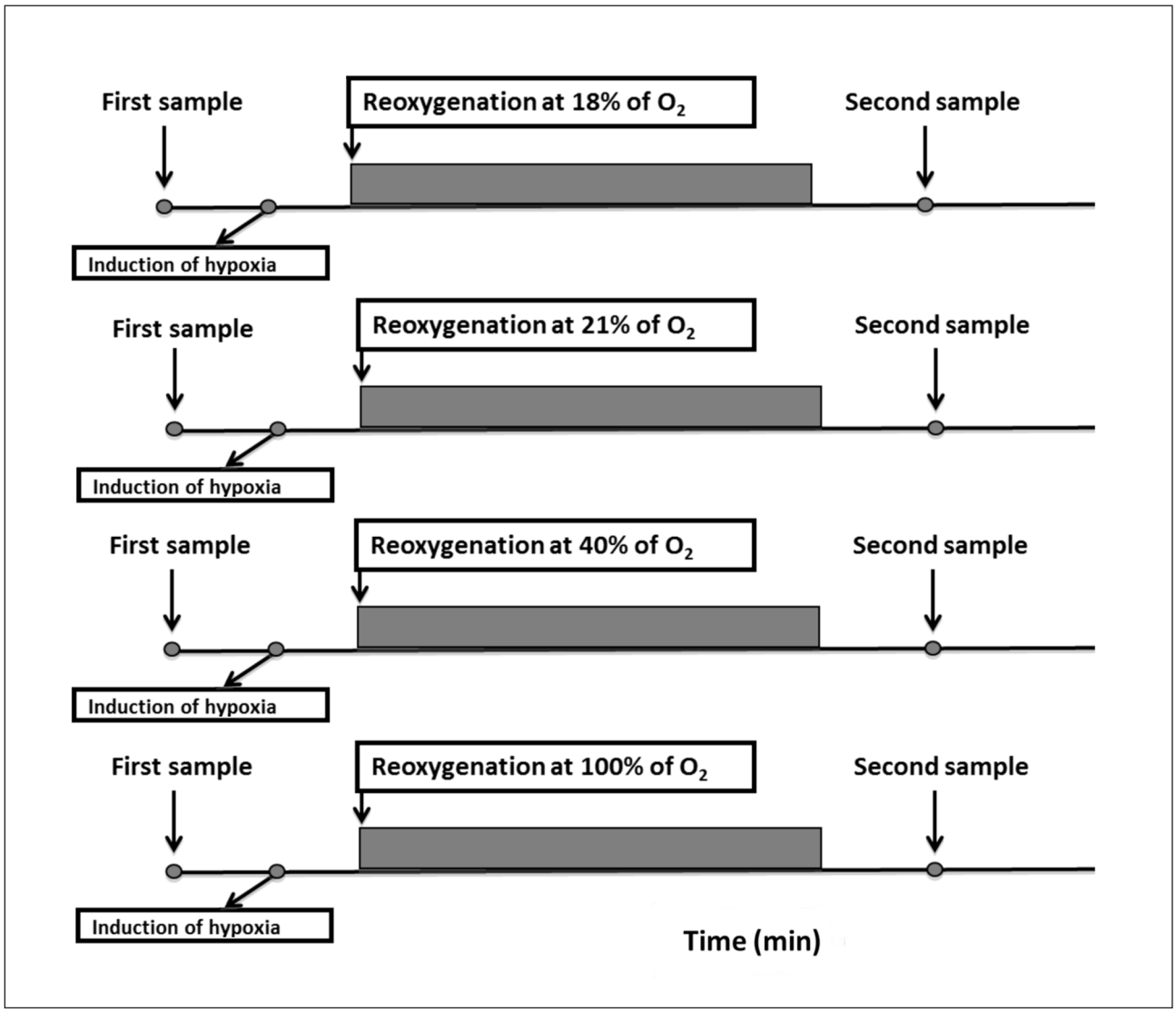

Figure 1. Experimental model: the first sample was collected before the induction of hypoxia, the second one after reoxigenation.

delay of 2 s, and 128 scans. The residual water signal was suppressed by applying a pre saturation technique with low power radiofrequency irradiation for 2 s during relaxation. The total acquisition time was of 8 min. Chemical shifts were referred to the TSP single resonance at 0.00 ppm.

Data processing and multivariate analysis

Each spectrum was divided into consecutive "bins" of 0.04 ppm. The spectral area investigated was the region between 0.4 and 9.6 ppm. The regions between 4.68 and 5.14 ppm and 5.48 and 5.90 ppm were excluded in order to remove variations in the presaturation of the residual water resonance and the signal of urea. In order to minimize the effects of different concentrations of urine samples, the integrated area within each bin was normalized to a constant sum of 100. The final data set consisted in a matrix of 208X40 values. Columns represented the normalized area of each bin (variables) and rows represented samples (subjects). Multivariate statistical analysis was performed on the matrix generated using SIMCA-P+ software (version 13.0, Umetrics; Umea; Sweden). NMR's variables were scaled using Pareto scaling in order to emphasize all metabolite signals and reduce the noise in the spectrum. Partial Least Square Discriminant Analysis (PLS-DA) were applied for maximize the discrimination between samples. In order to evaluated the goodness of the models, the variance and the predictive ability (R2X, R2Y, Q2) were calculated. The most significantly variables were extracted by the loading plot of the model. These variables were quantified using Chenomx NMR Suite 7.1 [6, 7]. The U Mann Whitney test was applied to underline the significant changes in the concentrations of discriminating metabolites, (version 6.0, Statsoft, Bedford UK).

Results

The time of resuscitation after the hypoxia treatment was as follows: for the 18%, 21%, 40% and 100% group it was 14.5 ± 7.7 min, 33.7 ± 21.04 min, 76.5 ± 44.1 min and 57 ± 44 min respectively. As it resulted from the average times of recovery, the piglets reanimated with low concentration of oxygen (18% and 21%) had faster time of recovery while those reanimated with high concentration (40% and 100%) showed long time of recovery. As a consequence of the different time of resuscitation, a metabolomics analysis between the two classes of reoxygenated piglets with the slowest and fastest recovery was carried out: first group (4 piglets) RT < 15 minutes and second group (6 piglets) RT > 68 minutes (**Fig. 2**). In order to identify the variables (metabolites) separating the two groups of piglets the spectral data of the corresponding urine samples were subjected to the bucketing procedure, and the resulting data were analyzed using a PLS-DA classification model.

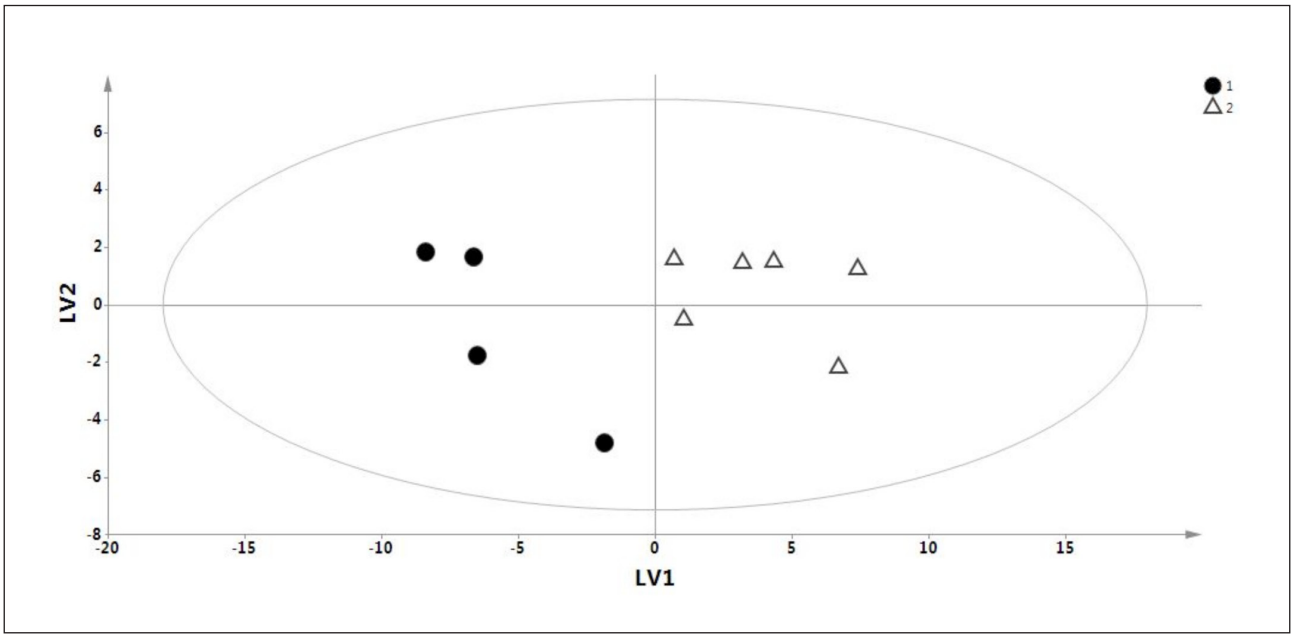

Figure 2. A PLS-DA model between piglets with RT < 15' (black circles) and piglets with RT > 68' (open triangles). Model parameters for the explained variation (R2X and R2Y), and the predictive capability, Q2, were: R2X = 0.789; R2Y = 0.869; Q2 = 0.689.

The prediction model yields R2X = 0.789; R2Y = 0.869; Q2 = 0.689. The ^{1}H-NMR chemical shifts of the identified metabolites are shown in **Tab. 1**. The metabolites significantly contributing to the separation between the groups were: glucose (increased in the class with RT > 68') acetoacetate, alanine, succinate, dimethylamine, methanol, N-phenylacetylglycine, sarcosine (increased in the class with RT < 15'). The quantification of these metabolites was performed as shown in **Fig. 3** and **Tab. 2**.

It was not possible to quantify TMAO and betaine because their signals overlap at 3.27 ppm in the NMR spectrum.

Discussion

In the present study, we performed a ^{1}H-NMR metabolomics analysis on urine from an experimental model of newborn piglets subjected to hypoxia and reoxygenation using 4 different oxygen concentrations. The piglet model appears to be a good model to extrapolate information about human neonatal conditions [7, 8]. In fact, our model mimics the perinatal asphyxia events, which occur in about 4 millions newborn worldwide [9], underlining the importance of the treatment. The primary effect of the hypoxia condition is a dramatic drop in mitochondria activity. Physiologically, the cell is forced to depend on glycolysis for ATP, but under stress conditions it may be not enough. The loss of ATP and the increases of acidosis may cause damage by a number of mechanisms and imbalances, and the system responds with compensatory mechanisms [10-12]. In our model we were able to describe a small portion of the metabolic aspects of this adaptation through the use of the ^{1}H-NMR technique. This technique creates a wide spectrum of signals belonging to different classes of molecules reflecting the activity of a biological system. An explanatory mechanism of few metabolites (important for our analysis) was performed (**Fig. 4**). The presence of acetoacetate and succinate suggest an energetic shift, which may help to compensate a reduced level of ATP (due to the hypoxia and resuscitation condition) and to reach more ATP production. Acetoacetate is a ketone body that is produced from acetyl-CoA during the ketogenesis process mainly in the mitochondrial matrix of hepatocytes. In normal conditions the metabolism of fatty acids takes place simultaneously to the metabolism of glucose, which is essential for

Table 1. ^{1}H-NMR chemical shift of the most significant metabolites identified in the first (< 15') and second group (> 68') of piglets.

Compound	Group	^{1}H (ppm)[a]	^{1}H Multiplicity[b]
Acetoacetate	γCH_3	2.28	s
	αCH_2	3.4	s
Alanine	βCH_3	1.49	d
	αCH	3.80	q
Sarcosine	$N\text{-}CH_3$	2.73	s
	αCH_2	2.60	s
Dimethylamine	$N(CH_3)_2$	2.72	s
Succinate	CH_2	2.41	s
Glucose	C_2H	3.23, 3.52	dd
	C_3H, C_6H C_5H C_4H C_5H, C_6H C_6H C_1H	3.73 3.46 3.40 3.82 3.88 5.24, 4.64	m m m m dd d
N-phenylacetylglycine	CH_2 $N\text{-}CH_2$	3.66 3.75	s d
	CH_2 $N\text{-}CH_2$ C_2H, C_6H C_4H C_3H, C_5H	3.66 3.75 7.34 7.37 7.41	s d m m s
Methanol	CH_3	3.36	s

[a] ^{1}H chemical shift are reported with respect to TSP signal (0.00 ppm).
[b] Multiplicity definitions: s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublets; m, multiplet.

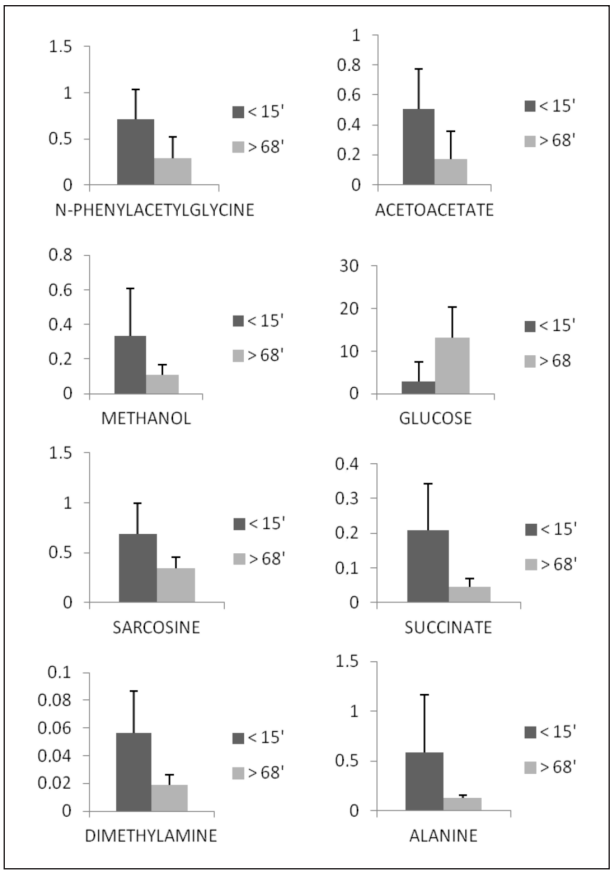

Figure 3. Quantifications of the most significant metabolites (U Mann Whitney test).

the formation of oxaloacetic acid. The latter reacts with the acetyl-CoA forming citrate, which is part of the Krebs cycle. In absence of glucose the levels of oxaloacetate is low and two molecules of acetyl-CoA will synthetize one molecule of acetoacetyl-CoA. Subsequently a reaction between acetoacetyl-CoA and another molecule of acetyl-CoA will form the β-hydroxy-β-methylglutaryl-CoA realising acetoacetate that is increased in the group with faster recovery underlining the alternative production of energy. The acetoacetate can be further reduced to beta-hydroxybutyrate or decarboxylated to acetone. Ketone bodies are then distributed to other tissues, where acetoacetate and beta-hydroxybutyrate can be converted to acetyl-CoA to give energy with a release of succinate that was increased in the faster recovery group. Further analysis are needed to better elucidate the role and mechanism of the other metabolites.

Conclusion

The metabolomic approach here proposed clearly identifies two different metabolic signatures related to the different oxygen concentration treatment supported to the animals. These differences underline the existences of complex metabolic pathways leading from the original hypoxic insult to the recovery outcome and they may also support the better result obtained with room air oxygen concentration.

What clearly appears is the basal presence of a basal noteworthy interindividual variability among piglets. Among them, the dynamic range of the metabolome is quite different from each other and these differences are accentuated when faced with important changes, such as asphyxia [12, 13]. These differences could help to understand why some piglets die [7], and some others survive, and why, among survivors, some present a rapid recovery time (< 15 min) and some others a long recovery time (> 68 min).

A better comprehension of all these mechanisms will allow a more individualized approach, eventually leading to different resuscitation strategies, in order to improve the outcome, especially in piglets with less basal energy before asphyxia and/or less resilience.

Table 2. Summary of the different urinary metabolic profile for the two groups.

Metabolites	Fold change (<15'/>68')	p
Glucose	–4.55	0.03
Sarcosine	2	0.01
N-phenylacetylglycine	2.44	0.05
Dimethylamine	2.92	0.01
Acetoacetate	2.94	0.045
Methanol	3	0.03
Succinate	4.52	0.01
Alanine	4.65	0.01

Declaration of interest

The Authors declare that there is no conflict of interest.

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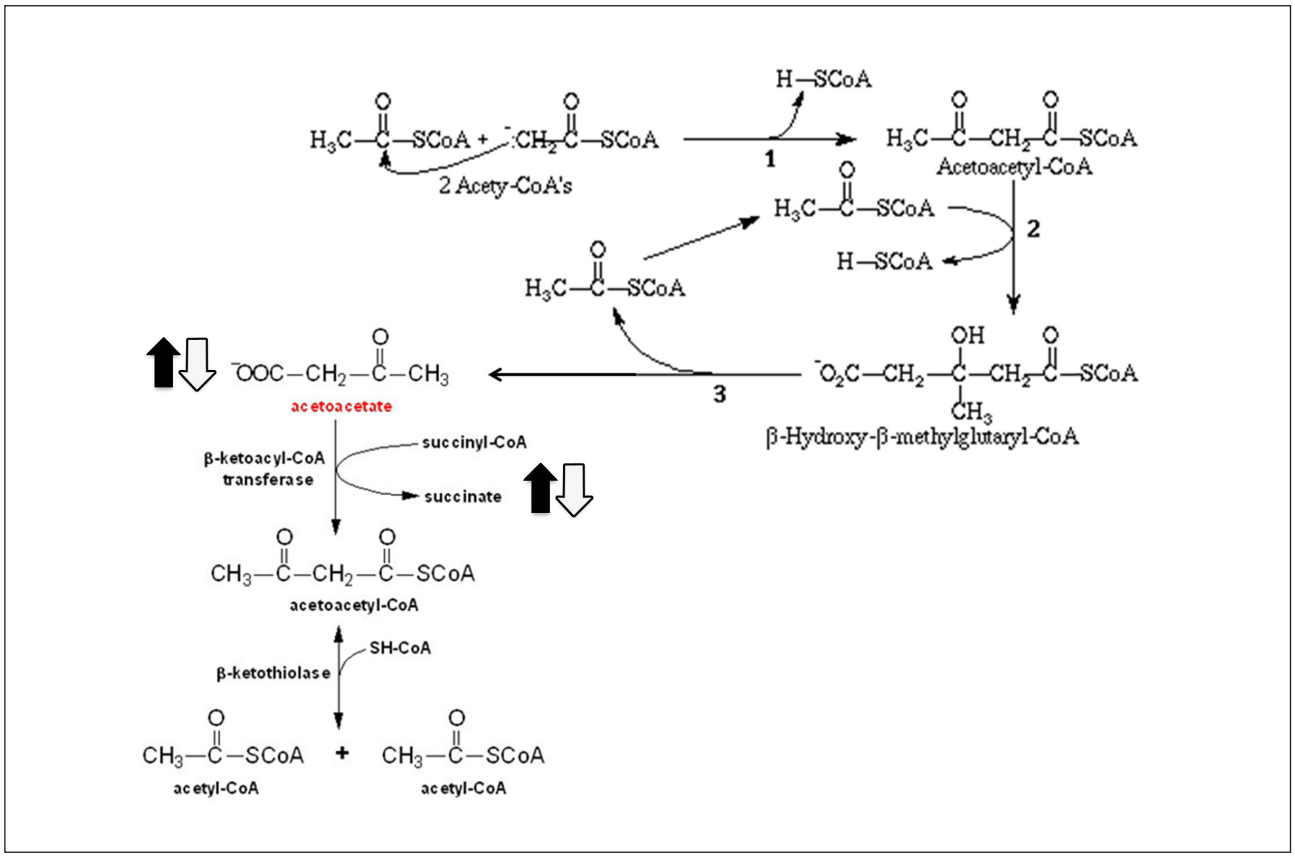

Figure 4. Metabolic pathway of the ketogenesis: black arrows represent metabolites increased in the group with RT < 15', white arrows metabolites decreased in the group with RT> 68'.

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