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The effect of rejuvenation of aged erythrocytes on biochemical parameters in the perfused hind limb muscle preparation

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(1) A systematic investigation was carried out into the use of time-expired erythrocytes in an isolated perfused skeletal muscle preparation. Comparisons were made between erythrocytes subjected to a process of 'rejuvenation' (Rennie and Holloszy (1977), Biochem. J. 168, 161–170) and untreated erythrocytes (controls). (2) The use of rejuvenated erythrocytes had no significant effect on concentrations of muscle ATP, phosphocreatine and lactate, nor fractional rates of muscle protein synthesis. However, muscle water concentrations were reduced when compared to controls. (3) There was an influx of K^+ from the plasma into rejuvenated erythrocytes. This was accompanied by a substantial loss (17%) of intramuscular K^+. There was also loss of K^+ from control preparations but this amounted to approx. 1% of muscle content. (4) Erythrocyte fragility was greater in the control perfusate (6%, haemolysis) when compared to the medium with rejuvenated cells (1%, haemolysis). As a consequence of either erythrocyte storage, rejuvenation or haemolysis, plasma concentrations of phosphate, magnesium, calcium and potassium were significantly different from starting values, by as much as 300% in both groups, and varied throughout the study. (5) It is concluded that the use of rejuvenated erythrocytes does not confer any advantage in unexercised perfused skeletal muscle preparations. However, both types of erythrocyte induce changes in perfusate composition relative to starting or in vivo profiles.

Introduction

Aged (time-expired) human erythrocytes have been used as the principal oxygen carrier in numerous perfused skeletal muscle preparations for example those by Ruderman et al. [1] Caldwell et al. [2] and Kemmer et al. [3]. Compared with freshly donated cells, they are readily available and have minimum rates of lactate release. However, one effect of erythrocyte storage is an increased affinity for oxygen, i.e., the oxygen dissociation curve shifts to the left, [4–6]. It has been claimed that isolated perfused rat skeletal muscle takes up less oxygen with aged erythrocytes, when compared with fresh erythrocytes [7]. This implies muscle dysfunction because of hypoxia. By a process of 'rejuvenation' the affinity of haemoglobin for oxygen can be reduced [8]. Rennie and Holloszy [7] used this technique and suggested that

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oxygen uptake in perfused muscle was greater with rejuvenated erythrocytes. On this basis, the rejuvenation procedure has been applied to a very large number of perfusion studies. However, there has been no systematic evaluation of the perfusion medium, i.e., comparing the same biochemical indices, in the same set of animals, under identical conditions. The experiments below describe a systematic investigation of aged and rejuvenated-aged erythrocytes.

Methods and Materials

Male Wistar rats were purchased from Charles River, (Margate, Kent, U.K.) and maintained in a humidified and temperature controlled environment, on a 12 h-light: 12 h-dark cycle, commencing at 08.00 h. Rats were fed ad libitum on a synthetic powdered diet, containing 20% (w/w) protein [9] until they were 165–190 g body weight. [U-^{14}C]Tyrosine was obtained from Amersham International (Amersham, Bucks, U.K.). Sources of other materials were as described previously [10–12].

Method of perfusion

Perfusion medium and rejuvenation of erythrocytes. Packs of time-expired human erythrocytes were obtained from the local Blood Transfusion Centre (Edgeware, Middlesex), and used between 36–40 days (mean, 37 days) from the donation date. They were stored at 0–4° C prior to use. The packs were alternately designated for 'rejuvenation' or used in the usual way [10–12]. Each pack (approx. 400 cm^3) of untreated (control) erythrocytes was 'washed' with bicarbonate buffer (containing 1 IU/ml heparin). This involved mixing the erythrocytes gently with 800 ml bicarbonate buffer, centrifuging (at $300 \times g$ for 20 min at 25° C), then aspirating the supernatant and all non-erythrocyte material. The procedure was repeated a total of three times. Rejuvenated erythrocytes were subjected to the following procedure [7,8]: each pack of aged cells was gently added to an equal volume of bicarbonate buffer pH 7.45 containing 1 IU heparin/ml. In a 1 litre centrifuge bucket 50 ml of the rejuvenation solution (50 mmol/l pyruvic acid, 50 mmol/l inosine, 50 mmol/l sodium phosphate, 100 mmol/l glucose, 5 mmol/l adenine, 0.15 mmol/l NaCl) was added to the cells and the contents were incubated at 37° C with mild agitation. At the end of 1 h the bucket was centrifuged ($3000 \times g$ for 20 min at 25° C). The supernatant and all non-erythrocyte material were gently aspirated. Subsequent steps were identical to untreated (control) erythrocytes.

The perfusion medium, in bicarbonate buffer, contained the following: 45% (v/v) erythrocytes containing 12–16 g haemoglobin per 100 ml of perfusate, 5.25% (w/v) dialysed bovine serum albumin (Cohn Fraction V), 11 mM glucose, 0.04 mM pyruvate, 1 IU/ml heparin, 25 mIU/ml insulin and normal concentrations of amino acids, as determined in vivo [13]. As soon as the perfusate was made, and immediately after the pH was adjusted, a sample of the medium was taken for measurement of 'pre-perfusion' values. At this point 135 ml perfusate was added to the recirculating perfusion circuit at 37.5° C. At the same time, 50 ml of perfusate was placed in the prefusion cabinet (37.5° C) for non-circulating samples. Approx. 60–90 min elapsed between taking the 'pre-perfusion' sample and completion of the washout period (time, 0 min, see below).

Perfusion procedure. This has been described previously [10–12]. Subsequent to aortic procedure cannulation and hemisection above the diaphragm, the preparation was covered in plastic film, transferred to a temperature-controlled cabinet (37.5° C) and flushed with 50 ml of non-recirculating medium at a rate of 10 ml/min. At the end of the washout period (time, 0 min) a recirculating medium (85 ml) was used. At 50 min, the left common iliac vessels were ligated and the left leg was amputated to obtain intermediate samples; perfusate flow was reduced according to the remaining mass of tissue for each preparation. At 90 min experiments were terminated.

Measurement of protein synthesis

At 15 min, 38 ml of recirculating medium was replaced with fresh medium containing a high concentration (4 μCi; 28 μmol) of L-[U-^{14}C]tyrosine [11]. Fractional rates of protein synthesis (defined as the percentage of tissue protein, renewed each day by synthesis, i.e., %/day), was

calculated from the formula:

$$k_s = \frac{S_B \times 100}{S_i \times t} (\%/\text{day})$$

where S_B and S_i are the specific radioactivity of bound tyrosine in tissue protein, and free tyrosine in tissue homogenate, respectively and 't' is the incorporation period in days. Perfusions with untreated or rejuvenated erythrocytes, measurement of synthesis, analysis of tissue and perfusate samples were carried out concurrently (see below).

Measurement of tissue and perfusate metabolites

At 50 or 90 min, muscles were exposed, frozen between aluminium blocks (previously cooled in liquid nitrogen), and stored at −196°C prior to analysis. Muscle for potassium (K^+) estimation was solubilized by dissolving in concentrated nitric acid prior to assay. Other estimations of ions were made on plasma. Lactate measurements were made on whole perfusate precipitated directly in 0.9 M $HClO_4$.

For tissue metabolites, frozen muscles were powdered, precipitated in 0.9 M $HClO_4$ and the supernatants were neutralised with 4 M KOH. The following assays were used: K^+ [14], magnesium [15], calcium [16], inorganic phosphate [17], plasma and whole perfusate haemoglobin [18], lactate [19], ATP and phosphocreatine [20], amino acids by ion-exchange chromatography [13], water content by drying to constant weight and RNA and protein [21].

Presentation of data

Data relating to perfusions which did not fall within the confines of criteria described by Preedy et al. [12] were rejected from analysis. All data are presented as mean ± S.E.M. with the number of observations in parentheses. Differences were assessed with Student's t-test, with significance determined if $P < 0.05$. Paired statistics were used where appropriate.

Results

Table I shows that the concentrations of ATP and phosphocreatine in muscle from control perfusions were not significantly different from values for preparations using rejuvenated cells. The water content of muscle, however, was lower when rejuvenated cells were used. Concentrations of lactate in muscle were also lower with the rejuvenated group, though this was not statistically significant (Table I). This may have reflected the reduced lactate concentration in the whole perfusate of the rejuvenated group at the beginning of perfusion (2.08 ± 0.26(4) μmol/ml and 0.96 ± 0.20(4) μmol/ml in the control and rejuvenated group, respectively, $P < 0.02$). Presumably, this

TABLE I

EFFECT OF USING TIME-EXPIRED AND REJUVENATED TIME-EXPIRED ERYTHROCYTES ON THE CONCENTRATION OF MUSCLE ATP, PHOSPHOCREATINE, LACTATE AND WATER, AND RATES OF PROTEIN SYNTHESIS

Perfusions were prepared from fed male rats (165–190 g body weight) as described in Materials and Methods. The perfusion medium contained time-expired human erythrocytes (control) or time-expired human erythrocytes subjected to a process of 'rejuvenation'. Analysis for tissue metabolites were made on quadriceps (ATP, phosphocreatine, lactate) or tibialis anterior muscles (water) taken at the end of the experiment (90 min). Rates of protein synthesis were measured in gastrocnemius or psoas muscles between 15 and 90 min. DIfferences between control and rejuvenated groups (four observations were made for each group, except * data were $n = 3$), [a] $P < 0.001$. There was no other statistical difference (i.e., $P > 0.05$) for other parameters.

	Control	Rejuvenated
ATP (μmol/g wet wt.)	7.80 ± 0.52	7.90 ± 0.90
Phosphocreatine (μmol/g wet wt.)	6.81 ± 1.19	8.68 ± 1.41
Lactate (μmol/g wet wt.)	10.09 ± 1.41	7.56 ± 1.47
Water (% weight/wt.)	77.04 ± 0.13	75.93 ± 0.16 [a]
Fractional rate of protein synthesis (%/day)		
Gastrocnemius	10.51 ± 0.83 *	11.49 ± 0.57
Psoas	10.05 ± 0.71 *	10.22 ± 0.31
Synthesis/RNA (g protein/g RNA per day)		
Gastrocnemius	10.78 ± 0.89 *	10.96 ± 0.94
Psoas	11.20 ± 0.93 *	11.24 ± 0.93

resulted from differences in the preparation of the cells, such as improved washing, or transport of lactate out of the erythrocyte during rejuvenation. Rates of protein synthesis in gastrocnemius and psoas muscles are also displayed in Table I. There was no difference in fractional synthesis rates, nor the amount of protein synthesised per unit RNA.

Table II shows levels of plasma haemoglobin in non-recirculating and recirculating perfusate, expressed as a percentage of haemoglobin in whole perfusate. With control cells there was spontaneous haemolysis, as indicated by release of free haemoglobin during the perfusion. This rate was not increased by the perfusion procedure and at the end of treatment approx. 6% of total erythrocytes had haemolysed. With rejuvenated cells, the perfusion procedure also had no additional effect on the spontaneous rate of haemolysis, but the value (1% of total cells) was less than for unprocessed cells. The concentrations of various ions are also displayed in Table II. We assumed the characteristics of uptake or release of ions in non-circulating medium were similar to that in recirculating medium. Thus, differences in con-

TABLE II

THE EFFECT OF USING TIME-EXPIRED AND REJUVENATED TIME-EXPIRED ERYTHROCYTES ON HAEMOLYSIS AND PLASMA ION CONCENTRATIONS

Preparation of the control and rejuvenated medium is described in Materials and Methods. 'Starting' concentrations refer to calculated amounts, whilst 'pre-perfusion' values refer to samples taken shortly after making up the perfusion medium. Recirculating values refer to the medium in the presence of the hemicorpus, whilst the non-circulating data pertains to medium placed in a glass vessel inside the perfusion cabinet. Differences between appropriate control ($n = 4$) and rejuvenated ($n = 4$) groups at corresponding time points; [a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$. Differences between non-circulating and recirculating medium at the corresponding time points (paired t-tests); [d] $P < 0.05$; [e] $P < 0.01$; [f] $P < 0.001$.

	Min	Control		Rejuvenated	
		non-circulating reservoir	recirculating perfusate	non-circulating reservoir	recirculating perfusate
Haemolysed erythrocytes (%)	pre perfusion	0.17±0.02	–	0.15±0.07	–
(plasma haemoglobin/total	0	2.96±0.44	3.17±0.41	0.36±0.07 [c]	0.44±0.14 [c]
medium haemoglobin)	50	–	5.08±0.47	–	0.84±0.15 [c]
	90	6.00±0.85	6.08±0.48	1.22±0.23 [c]	1.19±0.13 [c]
	starting	1.20			
Inorganic phosphate	pre-perfusion	1.51±0.08	–	0.88±0.05 [c]	–
(mmol/l)	0	4.40±0.24	4.10±0.28	2.22±0.30 [b]	2.16±0.18 [b]
	50	–	4.50±0.19	–	2.25±0.34 [b]
	90	4.83±0.28	4.48±0.21	2.55±0.09 [c]	2.65±0.15 [c]
Magnesium					
(mmol/l)	starting	1.19			
	pre-perfusion	1.50±0.06	–	1.47±0.01	–
	0	1.59±0.01	1.59±0.03	1.61±0.05	1.60±0.01
	50	–	1.47±0.03	–	1.42±0.04
	90	1.67±0.04	1.49±0.05 [e]	1.53±0.03 [a]	1.36±0.02 [f]
Calcium	starting	2.54			
(mmol/l)	pre-perfusion	3.73±0.11	–	2.93±0.18 [b]	–
	0	3.88±0.05	3.73±0.03	3.54±0.36	3.80±0.18
	50	–	3.34±0.09	–	3.53±0.13
	90	3.53±0.03	3.10±0.10 [d]	3.48±0.20	3.37±0.06
Lactate	Pre-perfusion	2.18±0.41	–	1.22±0.28	–
(μmol/ml)	0	2.22±0.17	2.08±0.26	1.19±0.20 [b]	0.96±0.20 [d]
	50	2.36±0.42	2.19±0.19	2.03±0.39	2.10±0.37
	90	3.03±0.30	3.29±0.21	2.58±0.07	2.84±0.17

centration, between non-circulating and recirculating medium may have reflected metabolism by the hemicorpus. Plasma inorganic phosphate in control and rejuvenated perfusate increased in both the absence and presence of perfused tissue. However, the plasma levels in the medium containing rejuvenated erythrocytes were between 10% and 50% greater than that in unprocessed cells. There was no suggestion of uptake or release of inorganic phosphate by the perfused tissue as determined by paired analysis between non- and re-circulating media.

Concentrations of magnesium and calcium in the re-circulating plasma were not affected by rejuvenation. In the non-circulating medium there was a tendency for magnesium and calcium to be slightly lower for rejuvenated cells. However, paired comparisons between non-circulating and circulating medium with either rejuvenated or control cells suggested that the hemicorpus took up magnesium. There was also an apparent influx of calcium in hemicorpuses perfused with untreated erythrocytes, but this was not observed in the rejuvenated group.

Table III shows plasma, whole perfusate and tissue concentrations of potassium. In both circulating and non-circulating medium, erythrocytes from the control group released K^+ into the plasma. This was partly due to the rate of haemolysis (Table II). Differences between the reservoir and recirculating medium indicate a small efflux of K^+ from the hind-limb preparation also. In contrast, in the rejuvenated group there was an influx of K^+ from the plasma into the erythrocytes of both recirculating and non-circulating medium. Differences between recirculating and non-recirculating medium were also indicative of tissue K^+ efflux. Analysis of whole perfusate confirmed a net increase in recirculating K^+ concentrations in the rejuvenated group, though this was not observed for untreated erythrocytes. There was also a decrease in muscle K^+ in the rejuvenated group, whereas muscle from control preparations maintained in vivo values throughout the 90 min.

TABLE III

THE EFFECT OF USING TIME-EXPIRED AND REJUVENATED TIME-EXPIRED ERYTHROCYTES ON PERFUSATE AND MUSCLE POTASSIUM

Experimental details were as described in Materials and Methods and in the legends to Tables I and II. Starting concentrations of muscle (tibialis anterior) potassium refer to in vivo samples, taken from the same set of animals as used for perfusions after anaesthesia and heparinisation. Differences between control ($n = 4$) and rejuvenated perfusions at comparable time points; [a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$. Differences between recirculating perfusate and non-circulating reservoir at corresponding time points; [d] $P < 0.01$; [e] $P < 0.001$. Differences between in vivo ($n = 4$) and in vitro values; [f] $P < 0.05$.

	min	Control		Rejuvenated	
		non-circulating reservoir	recirculating perfusate	non-circulating reservoir	recirculating perfusate
Perfusate	starting	5.95	–	5.95	–
Plasma	pre-perfusion	6.10 ± 0.08	–	5.46 ± 0.09 [b]	–
(μmol/ml)	0	7.24 ± 0.26	7.18 ± 0.30	4.23 ± 0.16 [c]	4.25 ± 0.15 [c]
	50	–	7.90 ± 0.15	–	5.18 ± 0.15 [c]
	90	7.89 ± 0.13	9.15 ± 0.08 [e]	3.26 ± 0.29 [c]	5.71 ± 0.17 [e,c]
Whole perfusate					
(μmol/ml)	0	27.2 ± 1.3	–	28.9 ± 0.5	–
	20	–	26.4 ± 0.4	–	27.1 ± 0.5
	90	27.8 ± 0.9	27.1 ± 0.8	28.7 ± 0.9	30.2 ± 0.3 [c,d]
Muscle					
(μmol/g dry wt.)	starting (in vivo)		315 ± 20		315 ± 20
	50		332 ± 30		312 ± 14 [a]
	90		312 ± 16		262 ± 8 [a,f]

Discussion

The basis of these experiments was the suggestion that the use of time-expired erythrocytes in perfused skeletal muscle preparations may result in reduced oxygen uptake, leading to a decline in metabolic integrity via hypoxia. In our previous studies, we demonstrated that protein synthesis and other metabolic indices in perfused muscle were sensitive to hypoxia in vitro [12]. By a process of rejuvenation, the oxygen-dissociation characteristics of the erythrocytes can be improved to increase oxygen uptake by skeletal muscle [7]. The results show that the use of rejuvenated erythrocytes alters neither ATP, phosphocreatine, lactate nor protein synthesis, parameters that in previous studies were shown to be useful indicators of viability in vitro [12]. By contrast, water content in the muscles from the rejuvenated group was lower, though we have been unable to corelate water content to muscle integrity or protein synthesis [12]. Further evidence to verify the metabolic viability of muscle perfused with unprocessed aged-erythrocytes was obtained from measurements of oxygen uptake. In hemicorpuses perfused under identical conditions to those reported here oxygen uptake was $32.8 \pm 2.8(7)$ μmol/h per g muscle [12]. This was comparable to a variety of values reported by Ruderman et al. [22] for other muscle perfusion studies (i.e., 13–40 μmol/h per g muscle).

The data in Table III show that during the experiment, K^+ was released by unprocessed time-expired cells, apparently almost entirely as a result of haemolysis. A haemolysis of 6.08% represents an increase of 1.69 nmol K^+/ml plasma, which is close to the observed value. The difference in $[K^+]$ between the reservoir and recirculating medium represented a small release of K^+ from the hemicorpus in control perfusions. If we assume that K^+ was derived exclusively from muscle, the efflux represented only 1% of the total muscle K^+. In contrast, in the rejuvenated group there was a continual influx of K^+ from plasma to erythrocytes of non-circulating medium. This was accompanied by first a decrease, and then an increase in the concentration of plasma K^+ of the recirculating medium. There was also an increase in total recirculating perfusate K^+, providing evidence that the influx from plasma to erythrocytes depleted the plasma, with a subsequent loss of muscle K^+. Direct estimation of muscle K^+ showed a 17% loss. Ward and Buttery [23] proposed that tissue dysfunction occurs when muscle loses 5% of K^+, although there was no evidence of dysfunction in the present studies.

The data in Table II show that abnormalities also occur in the concentrations of inorganic phosphate, calcium and magnesium. Moreover, although these measurements were made over 90 min, the changes were progressive and many perfusion studies last 3–4 h. In normal situations concentrations of individual plasma ions are confined to a narrow range, and a 50% decrease in plasma K^+, for example, represents a serious clinical crisis in man. We therefore felt that these results have important methodological implications. For example, muscle incubated in medium with low K^+ concentrations has been shown to have an altered response to hormones such as insulin [24] or adrenalin [25]. Also, ions such as potassium, calcium and magnesium are known regulators of protein turnover in various cell types including muscle [26–31]. However, the fundamental fact was that neither type of medium reflected the calculated (intended) ionic profiles. This problem was not improved by rejuvenation of the red cells, and for some ions it was aggravated. This could become important for studies designed to investigate the effect of altering concentrations of plasma ions.

In conclusion, the use of rejuvenated erythrocytes does not confer any advantage in perfused skeletal muscle, particularly with respect to ATP, phosphocreatine, lactate and protein synthesis. The results also indicate that both aged and rejuvenated erythrocytes induce changes in the perfusate composition that are unphysiological. Our data therefore conflict with the generally held belief that perfusion media containing erythrocytes mimic the blood of animals in vivo.

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