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Urinary excretion of exogenous glycerol administration at rest

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Since 2010, glycerol has been ruled a masking agent by the World Anti-Doping Agency and consequently its administration is prohibited in sports. A detection method is available but little is known about the urinary excretion following administration.

Fourteen well-trained cyclists (27.0 ± 5.4 years; $\dot{V}O_{2max}$: 63.9 ± 8.5 ml/kg/min) were administered glycerol (1 g/kg body mass + 25 ml water/kg body mass) and placebo (25 ml water/kg) in a cross-over study. Blood and urine samples were collected before administration and after 2.5, 4, and 6.5 h. Urine samples were further collected up to 24 h post-administration.

Following glycerol administration, urinary glycerol increased from 10.9 ± 15.5 to 50581 ± 23821 μg/ml within 2.5 h. In the placebo group, urinary glycerol did not exceed 26.8 ± 31.3 μg/ml. Urinary concentrations in the glycerol group were significantly higher than in the placebo group for 16.9 ± 1.0 h.

In comparison to placebo, glycerol caused a larger increase in body weight (0.69 ± 0.42 vs. 0.27 ± 0.44 kg; $p < 0.05$) and a reduced urine output (972 ± 379 vs. 1271 ± 387 ml; $p < 0.05$). Reductions in haemoglobin and haematocrit were significantly greater after glycerol (−0.60 ± 0.28 g/dl; -1.7 ± 0.7%) than after placebo administration (−0.29 ± 0.39 g/dl; -0.9 ± 1.1%).

The study shows that glycerol administration was detectable in urine for several hours. Even though there were significant reductions in haemoglobin and haematocrit after 2.5 h, the plasma expansion by glycerol appeared rather marginal in comparison to placebo.

Keywords: plasma expansion; fluid retention; haemoglobin; haematocrit; doping

Introduction

Glycerol is a three-carbon alcohol which primarily serves as the backbone of triacylglycerides. Plasma concentrations of glycerol are typically below 0.1 mmol/L[1] but may be elevated when lipolysis is stimulated, such as during prolonged fasting, exercise, or increased catecholamine levels.[2–4] Since January 2010, glycerol has been listed by the World Anti-Doping Agency (WADA) as a masking agent. Hence, the oral or intravenous administration of glycerol is prohibited in sports.[5]

For doping-control purposes, a sensitive detection method for urinary glycerol has been presented and validated by Thevis *et al*.[6] Based on the analysis of urinary concentrations from a large sample of routine doping-control urines, the authors concluded that the natural occurrence of traces of glycerol should not exceed 200 μg/ml (2.2 mmol/L).

The exogenous application of glycerol has been used for the treatment of intracranial and intraocular oedema.[7] When administered with excess fluid, a proportion of the fluid is retained. This hyperhydration has been reported to increase blood and plasma volume[8] and consequently reduce haemoglobin and haematocrit values.[9,10] In sports, hyperhydration with glycerol has been suggested to be beneficial in situations when fluid intake is limited or heat stress occurs.[11] Several studies have shown that glycerol hyperhydration improves endurance performance under heat conditions.[12–15] For such situations, glycerol doses as high as 1.2 g/kg in addition to approximately 25 ml/kg fluid have been recommended.[11]

Glycerol is metabolized by glycerol-kinase to glycerol 3-phosphate, which can either enter gluconeogenesis or be converted to carbon dioxide.[4] At low plasma concentrations, glycerol is usually reabsorbed almost entirely in the nephron,[16] but it can be excreted via urine at higher concentrations. According to a recent publication, the renal threshold for glycerol lies in the range of 0.3 mmol/L.[11]

Following exogenous glycerol administration, peak plasma concentrations lie in the range of 10–20 mmol/L[9,14,17–19] so that the renal threshold is exceeded. Urinary excretion has been measured in only a few studies and peak concentrations have been reported to be in the mg/ml-range,[9,18] but urinary concentrations have been assessed over a short period of time only, so it is not known for how long urinary excretion is elevated and thus detectable.

Therefore, the purpose of the present study was to evaluate the urinary excretion and metabolism of exogenous glycerol in a placebo-controlled cross-over study. Additionally, the effects of glycerol on fluid homeostasis and doping-relevant blood parameters such as haemoglobin and haematocrit were also investigated.

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Methods

Study participants

Fourteen well-trained cyclists and triathletes (12 male, 2 female) participated in the study. In average, the participants were 27.0 ± 5.4 years of age, 184 ± 8 cm tall, weighed 73.7 ± 8.1 kg and had a body fat percentage of 10.1 ± 4.9 %. Their maximal oxygen uptake ($\dot{V}O_{2max}$) was 63.9 ± 8.5 ml/kg/min. All participants gave their written consent prior to the start of the experiments.

Study protocol

A schematic diagram of the study protocol is displayed in Figure 1. Each subject completed two identical test days and had to consume glycerol and placebo solutions in a cross-over design. The order of the solutions was selected randomly. The two test days were separated by a washout period of at least five days.

The subjects followed a controlled diet and had to refrain from any kind of strenuous exercise on the day prior to each test day. They were instructed to drink sufficiently in order to be adequately hydrated before the start of the experiment. Caffeine-containing beverages were not allowed in the morning before each test day.

Upon arrival at our lab (t = 0 h), a spot urine sample was collected and the urine volume was recorded to the nearest 10 ml. A blood sample was drawn from the anticubal vein and body weight and body composition was assessed using a bioimpedance balance (Tanita BC 418 MA balance (Tanita, Amsterdam, the Netherlands)).

Thereafter the participants consumed either a glycerol solution (1 g/kg glycerol + 25 ml/kg 0.1% NaCl) or a placebo drink (25 ml/kg 0.1% NaCl + artificial sweetener on the basis of sodium cyclamate and saccharin (Natreen®, Sara Lee Coffee & Tea, Cologne, Germany), which were identical in taste and colour. The drinks had to be consumed within 20 min. Until the end of the controlled study period (t = 6.5 h), the participants did not consume additional drinks or foods and were limited to sedentary indoor activities.

Further urine and blood samples were collected and body weight was measured again after 2.5, 4 and 6.5 h post-administration. Participants were free to urinate between the fixed sampling time points but had to collect the sample and record the volume. After 6.5 h, participants were released but had to collect additional spot urine samples until the morning of the next day (approximately 24 h post-administration). Throughout the whole test day, weighing was performed in light underwear and always after urination. Blood and urine samples were stored at −20 °C until analysis. The study protocol was approved by our University's ethics committee.

Biochemical analyses

Urinary glycerol was assessed using the method described by Thevis *et al.*[6] on an Agilent 6890 gas chromatograph coupled to an Agilent 5973 mass spectrometer (both Agilent, Waldbronn, Germany). The limit of quantification was 0.9 μg/ml. Samples exceeding the working range (0.9–96 μg/ml) were diluted and reanalyzed. In order to account for volume-dependent sample dilution, raw urinary glycerol concentrations (c_{raw}) were corrected to a specific gravity of 1.02 g/ml according to Equation (1). Urine-specific gravity was measured on DMA 38 Density Meter (Anton Paar, Graz, Austria).

$$c_{corrected} = c_{raw} \cdot (1.02 - 1) \cdot \left(\text{specific gravity}_{sample} - 1\text{g/ml}\right)^{-1} \quad (1)$$

Plasma glycerol concentrations were determined photometrically with a commercially available kit (F6428, Sigma, St. Louis, MO, USA). Haemoglobin and haematocrit concentrations and reticulocyte percentage were measured on a Sysmex XT 2000i (Sysmex, Norderstedt, Germany) from EDTA-blood. The $OFF_{hr\ score}$ was derived from haemoglobin concentration and reticulocyte percentage as described by Gore *et al.*[20] Plasma volume was calculated according to Dill and Costill.[21]

Computations and statistical analyses

All statistical analyses were performed using R software version 2.12.0 (The R Foundation for Statistical Computing, 2010). If not mentioned otherwise, all values are reported as mean ± standard deviation. Data was tested for normality using the Shapiro-Wilk test. For normally distributed data, a two-way analysis of variance followed by Student's t-test was applied and the Mann-Whitney U-test was used for not normally distributed data. Paired tests were used for data collected at fixed time points (t = 0, 2.5, 4, 6.5), whereas for urine values voluntarily collected between intervals or after the monitoring period, we applied a non-paired test. Differences were considered to be statistically significant with a probability of error below 5% ($\alpha < 0.05$). When multiple tests were applied, the α-level was adjusted by Bonferroni-correction.

For pharmacokinetic modelling of urinary glycerol excretion, a first-order kinetic model was applied using specific gravity-corrected urinary glycerol over time. The time course of the model represents an exponential decay (Equation (2)) and is characterized

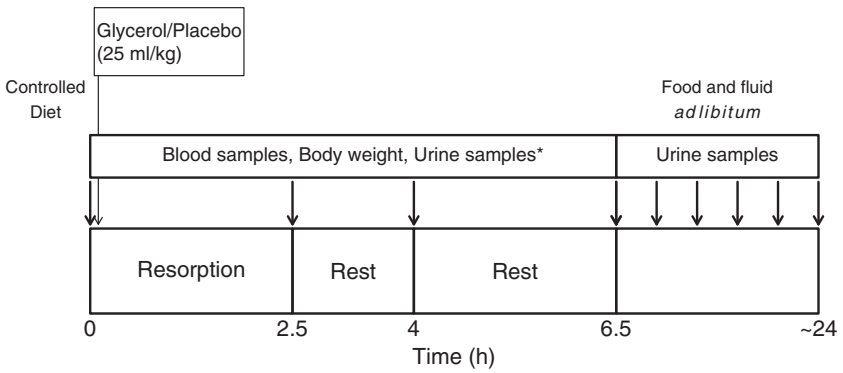

Figure 1. Schematic diagram of the study protocol.

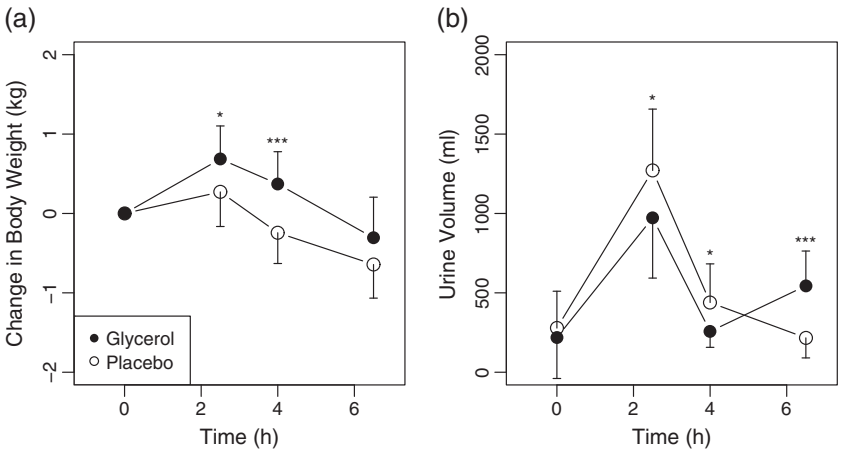

Figure 2. Changes in body weight (a) and urine production (b) following the administration of glycerol or placebo. Urine volume represents the total urine volume over the sampling interval.

by the elimination constant (k_{el}) and the initial urinary concentration (c_0):

$$c = c_0 \cdot e^{(-k_{el} \cdot t)} \quad (2)$$

C_0, k_{el} and the half-life ($t_{1/2} = \ln(2)/k_{el}$) were determined individually using linear regression following log-transformation. Only urines samples provided after the individual maximal concentration were included into the model. In order to account for continuous urine production, time (t) was expressed as the midpoint of each sampling interval.

Results

Hydration status

Glycerol administration caused a significant increase in body weight after 2.5 h (+0.69 ± 0.42 kg ($p < 0.001$). Body weight continually decreased thereafter (Figure 2a) but was still above the initial weight after 4 h (+0.37 ± 0.40; $p < 0.05$). In the placebo group, highest values were also observed after 2.5 h, but the increase was not significant (+0.27 ± 0.44 kg; $p = 0.11$).

The weight difference between glycerol and placebo administration was statistically significant at 2.5 and 4 h post administration but did not reach significance after 6.5 h ($p = 0.07$).

The ingestion of 25 ml/kg fluid caused a marked increase in urine production after 2.5 h in both groups (Figure 2b). Urine volume was significantly greater in the placebo group between 0 and 2.5 h and between 2.5 and 4 h. In the interval between 4 and 6.5 h, urine production was significantly greater in the glycerol group (544 ± 219 ml *vs* 216 ± 126 ml; $p < 0.001$).

Neither over the controlled study period of 6.5 h (1992 ± 733 ml (G) *vs* 2204 ± 669 ml (P), $p = 0.23$) nor over the whole collection period of approximately 24 h (3792 ± 1548 ml (G) *vs* 3715 ± 1214 ml, $p = 0.82$) was there a significant difference in the cumulative urine volume.

Blood parameters

After fluid administration, haemoglobin concentrations and haematocrit decreased in both groups (Figure 3), but the reduction was significantly larger in the glycerol group after 2.5 h. Both haematological parameters were significantly lower than initial values over the whole study period in both groups (Table 1).

Glycerol ingestion caused a plasma volume expansion of 7.5 ± 3.5% ($p < 0.001$) and plasma volume remained significantly elevated until 6.5 h. In the placebo group, plasma volume was

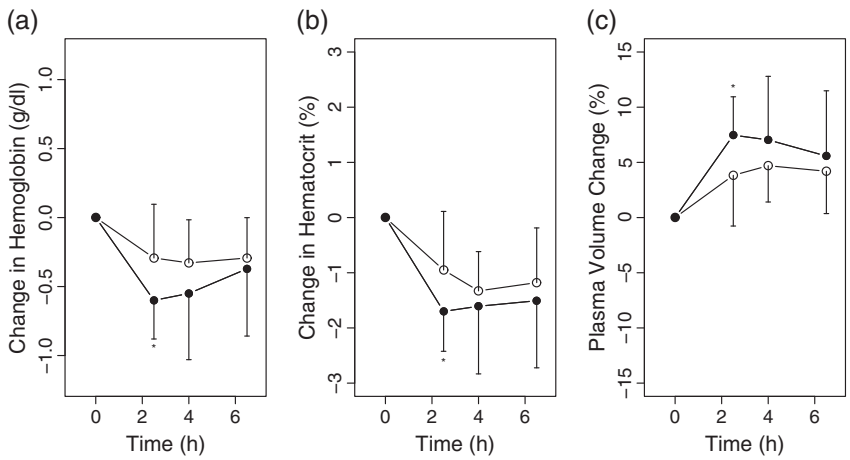

Figure 3. Changes in haemoglobin concentration (a), haematocrit values (b) and plasma volume (c) following the ingestion of glycerol (filled symbols) or placebo (open symbols).

Table 1. Absolute haemoglobin and haematocrit levels following the application of glycerol or placebo.

	Haemoglobin (g/dl)		Haematocrit (%)	
Time (h)	Glycerol	Placebo	Glycerol	Placebo
0	15.0 ± 0.8	14.7 ± 1.2	44.1 ± 1.9	43.6 ± 2.8
2.5	$14.4 \pm 1.0^{\#\#\#}$	$14.4 \pm 1.1^{\#}$	$42.4 \pm 2.2^{\#\#\#}$	$42.6 \pm 2.4^{\#}$
4	$14.4 \pm 1.1^{\#\#}$	$14.3 \pm 1.0^{\#\#}$	$42.5 \pm 2.5^{\#\#\#}$	$42.2 \pm 2.4^{\#\#\#}$
6.5	$14.6 \pm 1.1^{\#}$	$14.4 \pm 1.0^{\#\#}$	$42.6 \pm 2.6^{\#\#}$	$42.4 \pm 2.6^{\#\#\#}$

Significantly different from 0 h (# : $p < 0.05$,## : $p < 0.01$;### : $p < 0.001$)

also significantly above initial values over the whole period and peaked after 4 h (+4.7 ± 3.3 %). Plasma expansion was significantly larger in the glycerol group only after 2.5 h.

There were no significant differences in reticulocyte percentage and $OFF_{hr\ score}$ between glycerol and placebo administration at any time (data not shown). All samples in the present study were within the limits for reticulocytes and $OFF_{hr\ score}$ used for doping control purposes.[22]

Glycerol metabolism

Plasma glycerol concentrations (Figure 4) increased from baseline values (0.17 ± 0.12 mmol/L; 16 ± 11 μg/ml) to peak concentrations of 12.6 ± 5.3 mmol/L (1160 ± 488 μg/ml) after 2.5 h and decreased gradually thereafter. Plasma concentrations were still elevated after 6.5 h (1.25 ± 1.19 mmol/L; 115 ± 109 μg/ml, $p < 0.01$). In the placebo group, plasma concentrations remained fairly stable between 0.13 ± 0.10 mmol/L (12 ± 9 μg/ml, 0 h) and 0.10 ± 0.05 mmol/L (9 ± 5 μg/ml, 6.5 h). The difference between glycerol and placebo was significant for all three time points after glycerol ingestion.

Glycerol was rapidly excreted into urine. Specific gravity corrected urinary concentrations increased from 10.9 ± 15.5 μg/ml (0 h) to 38271 ± 19417 μg/ml in the first voluntary sample (1.4 ± 0.4 h post application) and peaked after 2.5 h (50581 ± 23821 μg/ml; Figure 4b). Following the 4 h-sample (47424 ± 20003 μg/ml), urinary concentrations decreased gradually but were still significantly higher than in the placebo group after 15 to 18 h (mean: 16.9 ± 1.0 h; 75 ± 67.9 μg/ml (G) *vs* 4.4 ± 2.5 μg/ml (P; $p < 0.05$).

In the control group, urinary glycerol concentrations were in the low μg/ml-range over the whole study period. The highest mean concentration was observed at 2.5 h post application (26.8 ± 31.3 μg/ml).

When all samples from the placebo group (n = 133) were pooled, the median for the corrected urinary concentration was 5.0 μg/ml. The 95% and 99.9% quantiles lay at 33.5 and 103 μg/ml. For uncorrected urinary concentrations, the median was 1.5 μg/ml; 95% of the values were below 10.3 μg/ml and 99.9% were below 60.3 μg/ml.

Pharmacokinetic model

The pharmacokinetic characteristics of glycerol excretion are displayed for each individual participant in Table 2. In average, the initial concentration c_0 was 185 ± 196 $\cdot 10^3$ μg/ml and the elimination constant was 12.7 ± 3.0 /day, resulting in a mean half-life of 1.38 ± 0.33 h (Table 2). The mean coefficient of determination (R^2) of the linear regression model was 0.83 ± 0.10.

Total urinary glycerol excretion over the collection period was 17.0 ± 5.4 g (range: 6.5–27.4 g), which corresponds to 23.4 ± 8.4 % of the initially administered dose (range: 10–46%).

Discussion

The goal of the present study was to investigate the urinary excretion of glycerol following the application of a typical dose, which could be used to affect plasma volume and haematological parameters. The study was placebo-controlled and was performed in a cross-over design.

Glycerol metabolism

Urinary glycerol excretion was rapidly increased and urine concentrations peaked between 30 000 and 50 000 μg/ml. In the few other studies which have reported urinary glycerol concentrations after administration of a similar dose, the peak values were between 19 and 28 mg/ml.[9,18]

Individual peak urinary concentrations were observed after 2.8 ± 0.8 h post administration (range: 2.0–4.0 h). Others have reported peak concentrations after 1 h following the administration of 1 g/kg[18] and after 1.5 h after the administration of 1.5 g/L body water.[9] After administration of 1.5 g/kg, highest urinary concentrations were reported after 4 h, when urine collection was stopped.[18] After approximately 4 h, urine concentrations decreased exponentially but were still significantly higher than in the placebo group until 15 to 18 h post administration (mean: 16.9 ± 1.0 h). Based on a one-compartment model, which explained 83 ± 10% of the variance in urinary glycerol excretion,

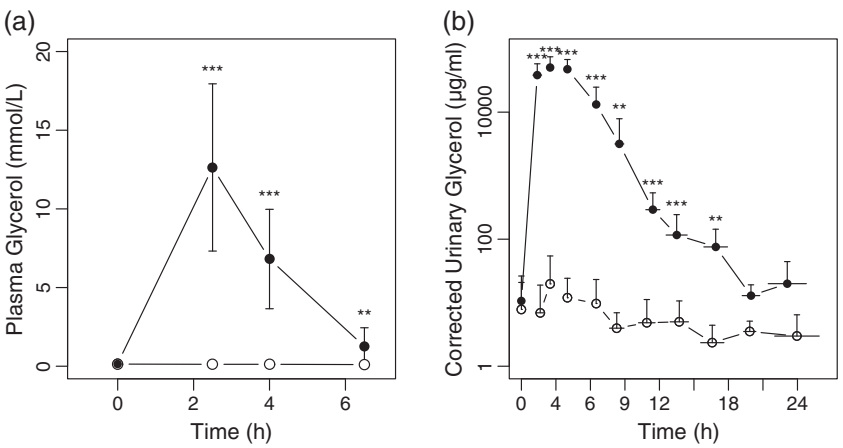

Figure 4. Plasma glycerol (a) and urinary glycerol concentrations (corrected for specific gravity; (b)) following the application of glycerol (filled symbols) or placebo (open symbols).

Table 2. Individual pharmacokinetic model of urinary glycerol excretion.

Participant	Time of concentration maximum (h)	Samples included	ln(C_0)	Initial concentration (10^3 μg/ml)	Elimination constant (/day)	Half-life (h)	R^2	Total excretion (g)	Total excretion % of dose
1	2.5	6	12.7	329	18.3	0.91	0.76	18.2	26
2	4.0	4	11.1	65	10.2	1.63	0.65	15.5	21
3	4.0	12	10.8	48	10.9	1.53	0.62	16.2	20
4	2.5	8	12.0	166	12.0	1.39	0.99	17.8	24
5	4.0	4	13.5	721	16.2	1.03	0.84	6.5	10
6	2.5	9	11.6	106	14.3	1.17	0.76	20.3	21
7	2.5	7	11.2	76	10.5	1.58	0.90	12.1	17
8	2.5	7	10.2	27	10.1	1.65	0.87	12.1	19
9	2.0	8	12.8	348	13.9	1.20	0.87	27.4	46
10	2.5	8	11.0	58	11.2	1.48	0.79	24.0	30
11	1.5	9	10.2	26	7.7	2.16	0.88	14.8	19
12	4.0	5	12.1	187	14.0	1.19	0.87	22.9	31
13	2.5	6	12.8	364	17.1	0.98	0.94	15.7	24
14	2.5	6	11.2	70	11.6	1.44	0.86	14.9	20
Overall	2.8 ± 0.8	7.1 ± 2.2	11.6 ± 1.0	185 ± 196	12.7 ± 3.0	1.38 ± 0.33	0.83 ± 0.10	17.0 ± 5.4	23.4 ± 8.4

urinary concentrations reached the threshold suggested by Thevis *et al*.[6] of 200 μg/ml after approximately 12 h.

Another possible elimination route of glycerol is the metabolism via the carbohydrate pathways,[4] but glycerol turnover has been reported to plateau when plasma concentrations exceed 1–2 mmol/L[23] so that urinary excretion becomes a major elimination route. In average, the total urinary glycerol excretion accounted for 23.4% of the administered dose (range: 17–46%). Freund *et al*.[9] estimated that 23% of a similar dose (1.5 g/L body water) were metabolized and 14.6% were excreted in the urine within 3 h post-administration.

In the placebo group, urinary glycerol concentrations remained in the low μg/ml range. Half of the urines in the control group did not exceed a concentration of 5.0 μg/ml and 99.9% of the urinary concentrations were below 103 μg/ml. To our knowledge, endogenous urinary glycerol concentrations have been analyzed so far only by Thevis *et al*.,[6] who reported that in about 50% of all analyzed doping control samples, the concentration was below 4.5 μg/ml and that urinary concentrations were between 60 and 140 μg/ml in only a small proportion of samples (1.3%).

Plasma glycerol concentrations indicate that glycerol was rapidly absorbed. Peak plasma concentrations in the range of 10–20 mmol/L (921–1842 μg/ml) were observed 2.5 h post-administration. In other studies, peak plasma concentrations between 8 and 13 mmol/L (737–1197 μg/ml) have been reported following the administration of a similar dose.[8,10,18,24] Plasma concentrations decreased continually but were still significantly elevated after 6.5 h.

Hydration status

The administration of 1 g glycerol per kg body weight in combination with fluid (25 ml/kg) caused a significantly larger fluid retention than the administration of fluid alone. In addition to the significant increase in body weight, fluid retention was also evident by the initial reduction in the urine output in the glycerol group, which has been reported in other studies.[9,10,14,18,25,26] The amount of fluid retained (approximately 400 ml) is in the range of other studies.[9,13,14,18,27]

Following the initial fluid retention between 0 and 4 h post administration, urine production was significantly larger in the glycerol group between 4 and 6.5 h, which indicates that the fluid-retaining properties of glycerol faded away. At the end of the urine collection period approximately 24 h post-administration, total urine volume was almost identical between glycerol and placebo administration (4226 *vs* 4144 ml). This is an agreement with results from Marino *et al*.,[28] who also reported that urine production was acutely reduced by glycerol, but that 24-h urine volume was not significantly different between glycerol and placebo administration.

It should be noted that there was considerable interindividual variability in the amount of fluid retention within our study, as the total amount of fluid retained within the first 2.5 h was in the range from 200 to 1500 ml in the glycerol group and −300 to 900 ml in the placebo group. This might in part be due to variability in the initial hydration status, even though all participants were asked to be well hydrated before the study start. However, there was no association between the initial urinary specific gravity and the amount of fluid retained in neither group (glycerol: $p = 0.79$; placebo: $p = 0.28$) and initial specific gravity was below 1.02 g/ml in all participants, so that all participants were considered adequately hydrated.[29]

Blood parameters

Glycerol administration led to a reduction of haemoglobin (−0.4 g/dl) and haematocrit values (−1.2%), even though both values were also affected by the administration of fluid alone. When compared with the control group, the differences in haemoglobin and haematocrit were significant only after 2.5 h. For all other sampling points, the differences were too small to be statistically significant. A significant reduction in haematocrit and haemoglobin values has also been found in several,[9,10,26] but not in all studies.[8,18,30]

Limitations of the study

The participants in our study were well-trained cyclists, but there was still considerable variance in their individual $\dot{V}O_{2max}$

(63.9 ± 8.5 ml/kg/min). We had to exclude several better trained cyclists from the study, because due to ethical considerations we could not administer a prohibited substance to athletes involved in a doping-control system.

Since plasma osmolality is affected by the administration of glycerol,[9] hematocrit values may have been measured erroneously with an electronic cell counter system. This may have also lead to erroneous values for the change in plasma volume, which were calculated with the Dill-Costill equation.[21] Nonetheless, we used the Sysmex system for the measurement of changes because it is routinely used for doping control purposes.

The present results are limited to a dose of 1.0 g glycerol per kg body weight and it remains unknown for how long smaller glycerol doses are detectable. According to Riedesel *et al*., a dose of 0.5 g/kg glycerol resulted in much smaller peak concentrations in serum (about 4 mmol/L) and urine (about 4000 μg/ml).[18] However, the administration of 0.5 g/kg did not affect haemoglobin and hematocrit significantly,[18] so that it seems unlikely that such a dose would be used to mask blood doping.

Glycerol may also be ingested from foods such as in wine, where concentrations as high as 10 g/L have been reported,[31] or as an additive to food, cosmetics or pharmaceuticals. The use of glycerol as a food additive is not restricted, so that theoretically it is possible for athletes to unintentionally ingest considerable amounts of glycerol. Further research is necessary to investigate whether these sources could lead to false-positive test results.

Conclusion

Even though glycerol caused a significantly higher fluid retention than fluid administration alone, our study indicates that the plasma-expanding properties and the effects on doping-relevant blood parameters are rather small.

Based on the results of the present study, the previously suggested urinary threshold of 200 μg/ml seems sufficient in order to detect the exogenous administration of glycerol for several hours, but further research is necessary to investigate whether this applies also under different conditions and at lower glycerol doses.

Acknowledgements

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