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Investigation of cavernosal smooth muscle dysfunction in low flow priapism using an *in vitro* model

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The effects of hypoxia (pO_2: 50 mmHg), acidosis (pH: 6.9) or glucopenia (absence of glucose) *in vitro* on the tone of the rabbit corpus cavernosum were investigated. The recovery of smooth muscle contractility following exposure to these conditions was also assessed. Hypoxia, acidosis or glucopenia alone or in combination showed a sustained reduction in the tone. Reperfusion of tissue strips showed complete recovery of smooth muscle tone for all conditions except when hypoxia and glucopenia were combined or when hypoxia, glucopenia and acidosis were used in combination. Incomplete recovery of tone was associated with a significant reduction in tissue ATP concentrations and an increase in the number of TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling)-positive nuclei. This indicates that following reversal of hypoxia, acidosis and glucopenia, failure of conventional α-adrenergic agonists to produce tumescence in low flow priapism is associated with irreversible smooth muscle cell dysfunction, which is linked to ATP reduction and smooth muscle cell death.
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Introduction

Priapism is defined as a prolonged penile erection that is maintained in the absence of sexual stimulation and persists despite orgasm. The most common subtype is low flow priapism, which is characterised by prolonged periods of stagnant ischaemia. In this situation, the cavernosal smooth muscle remains in contact with blood, which becomes progressively more hypoxic and acidotic. The pH of corporal blood aspirates can fall to 6.89 and the pO_2 can be as low as 15 mmHg (2 kPa).[1] In order to achieve penile detumescence, vasoconstriction of the resistance arterioles and contraction of the trabecular smooth muscle is required. Clinical observations indicate that as the duration of low flow priapism increases, the efficacy of pharmacological agents used to induce detumescence such as α-adrenergic agonists as well as surgically created shunts is significantly impaired.[2] Failure of detumescence in prolonged low flow priapism leads to irreversible smooth muscle dysfunction and is ultimately associated with long-term erectile dysfunction.[3]

Several investigators have described an *in vitro* model of low flow priapism utilising rabbit corpus cavernosum. These studies have investigated the effects of hypoxia, acidosis or glucopenia on cavernosal smooth muscle contraction.[4–8] The effects of acidosis and hypoxia have also been investigated using *in vivo* models.[9,10] As to which of these parameters is involved in irreversible smooth muscle dysfunction during low flow priapism has not yet been assessed.

Although the early stages of prolonged low flow priapism involve the development of hypoxia and acidosis in the ischaemic microenvironment, the later stages are characterised by the development of glucopenia due to substrate depletion.[11] A consequence of hypoxia, acidosis and glucopenia coexisting in the corporal microenvironment would result in the inhibition of the oxidative phosphorylation and the glycolytic pathway leading to ATP depletion. Hypoxia has been shown to induce ATP depletion in the corpus cavernosum.[5] In other

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smooth muscle preparations, glucopenia or hypoxia either alone or in combination have been shown to cause loss of tone and depletion of ATP.[12–14] Whether intracellular ATP levels can be maintained during ischaemia and subsequently replenished following reperfusion in the corpus cavernosum is not entirely clear.

We therefore investigated reversibility of smooth muscle dysfunction and ATP levels in low flow priapism by reproducing conditions of hypoxia, acidosis and glucopenia using an *in vitro* preparation.

Methods

Tissue procurement

Male white New Zealand rabbits (weight 3–4 kg) were housed in the Biological Services Unit between 16 and 19°C conforming to Home Office regulations. The rabbits were killed using an intravenous overdose of pentobarbitone into the marginal vein of the ear. The penis was identified and dissected to the level of the crural attachment to the ischium, where it was detached and immediately placed into Kreb's solution. The corpus cavernosum was sharply dissected from the tunica albuginea and two longitudinal strips were obtained. Each strip was then divided into two. All animal experiments were conducted according to the rules outlined by the Home Office, Animals (Scientific Procedures) Act 1986.

Organ bath experiments

Strips of rabbit corpus cavernosum ($3 \times 5\,mm^2$) were mounted horizontally in individual superfusion chambers between two ring electrodes, which were 4 mm in diameter. Each double-jacketed chamber was maintained at 37°C. The chambers were perfused with Kreb's solution at a constant flow rate of 1 ml/min using a Miniplus 2 (Gilson, Luton, UK) peristaltic pump. One end of the preparation was tied to an FT03C force displacement transducer (Grass Instruments, Quincy, Massachusetts, USA) connected to a Linearcorder WR 3101 (Graphtec, Tokyo, Japan) for recording isometric tension. The mechanical responses were also recorded on a computer by a specialised data-acquisition system (Axon Instruments, USA). Under normal conditions, the Kreb's solution was gassed with carbogen (95% O_2 and 5% CO_2). The preparations were stimulated electrically (electrical field stimulation; EFS) for 5 s with trains of rectangular pulses of 50 V, 0.3 ms pulse duration and at a range of frequency of 0.5–25 Hz, delivered by Grass S88 stimulators.

The preparations were given a tension of 0.6 g. They were then allowed to equilibrate for 60 min with carbogen. The test conditions were induced according to the protocols explained below, and the experiments were terminated by adding 50 μM prazosin to the Kreb's solution in order to identify the baseline.

The following conditions were used to reproduce the ischaemic parameters, which occur in low flow priapism:

Hypoxia. The gas mixture was exchanged from carbogen (95% O_2 and 5% CO_2) to one containing 95% N_2 and 5% CO_2 in order to mimic hypoxia. This resulted in a pO_2 of $\sim$50 mmHg (6.65 kPa) in the perfusion chamber.

Acidosis. The HCO_3^- composition of the Kreb's solution was reduced from 16.3 to 5 mM in order to reduce the pH to 6.9. The osmolarity was maintained by equimolar addition of NaCl such that the final concentration of NaCl was 144 mM. pH of the medium was periodically monitored using a pH meter.

Glucopenia. This condition was reproduced by utilising glucose-free Kreb's solution.

Experimental protocols

Protocol 1—Acute effect of hypoxia, acidosis and glucopenia on phenylephrine-induced tone: Tissue strips were precontracted with 20 μM phenylephrine (EC_{80}) following the equilibration period. The isometric tension was recorded simultaneously as conditions of hypoxia, acidosis or glucopenia or their various combinations were instilled.

Protocol 2—Time course for the recovery of phenylephrine-induced tone after the reversal of combination of hypoxia, acidosis and glucopenia: Tissue strips were precontracted with 20 μM phenylephrine following the equilibration period. The tension was recorded simultaneously as the tissue strips were superfused in a combination of hypoxia, acidosis and glucopenia. At various time points between 30 min to 6 h, the conditions were reversed using normal Kreb's solution containing 20 μM phenylephrine and gassed with carbogen. The recovered tone was expressed as the percentage of the initial tone. The tone from a control tissue, which was superfused with normal Kreb's solution was also recorded for up to 6 h to assess the spontaneous loss of tone.

Protocol 3—Recovery of phenylephrine-induced tone after the reversal of hypoxia, acidosis and glucopenia: Tissue strips were precontracted with 20 μM phenylephrine (EC_{80}) following the equilibra-

tion period. The isometric tension was recorded simultaneously as conditions of hypoxia, acidosis or glucopenia or their various combinations were instilled. The strips were exposed to these conditions for 4 h. The tone from a control tissue, which was superfused with normal Kreb's solution was also recorded to assess the spontaneous loss of tone during this time period. At the end of 4 h, the conditions were reversed using normal Kreb's solution containing 20 μM phenylephrine and gassed with carbogen. The recovered tone was expressed as the percentage of the initial tone.

Protocol 4—Cumulative concentration response to phenylephrine with and without hypoxia, acidosis and glucopenia: Tissue strips were divided randomly into two groups. The first group was superfused in normal Kreb's solution for 4 h. The second group was superfused with a combination of hypoxia, acidosis and glucopenia for 4 h. At the end of 4 h, both groups were superfused with normal Kreb's solution for 30 min. Then, increasing concentrations of phenylephrine were added (0.1–300 μM).

Protocol 5—Effect of hypoxia, acidosis and glucopenia on nitrergic responses: Tissue strips were precontracted with 20 μM phenylephrine. Noradrenergic and cholinergic blockade was performed using guanethidine (1 μM) and scopolamine (1 μM), respectively. EFS (50 V, 0.5–25 Hz, 0.3 ms pulse duration, for 5 s, every 2 min) was used to evoke nitrergic relaxations throughout the experimental period.[15] Tissue strips were then subjected to 4 h of a combination of hypoxia, acidosis and glucopenia. During this period, nitrergic relaxation responses were measured and their magnitude was expressed as a percentage of the tone. The nature of the relaxation responses was confirmed by the addition of an inhibitor of NO synthase (N^G-nitro-L-arginine methyl ester; L-NAME, 500 μM) or nerve blocker tetrodotoxin (TTX, 3 μM).

Measurement of ATP concentrations

Experiments were repeated where tissue strips underwent immediate snap freezing in liquid nitrogen at the following four time points: at the end of 60 min equilibration, after precontraction with phenylephrine (20 μM), following 4 h of combination of hypoxia, acidosis and glucopenia, and following 1 h of reperfusion with normal Kreb's solution. Four strips from the same animal were used for each time point and the experiment was repeated four times. The frozen tissues were crushed into a fine powder using a stainless-steel pestle and mortar on dry ice. A measure of 200 μl of 70% perchloric acid was added to the powder and vortex mixed vigorously. The preparation was then centrifuged for 10 min (13 000 *g*, 4°C). A measure of 150 μl of the supernatant was neutralised with 20 μl of 5 M K_2CO_3. After a further 10 min of centrifuging (13 000 *g*, 4°C), ATP was measured in duplicate in the supernatant using an ATP bioluminescent assay kit (Sigma, Poole, UK) according to the manufacturer's instructions. ATP standards (1 nM–10 μM; Sigma, UK) were used to construct a standard curve. Protein concentration in each sample was measured using Bio-Rad protein assay reagent (Bio-Rad, Hemel Hempstead, UK) based on the dye-binding procedure of Bradford.[16] The ATP concentration of each sample was expressed as pmol ATP/μg protein.

Histochemistry and immunofluorescence

Tissue strips were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer at four different time points: at the end of 60 min equilibration, after precontraction with phenylephrine (20 μM), following 4 h of combination of hypoxia, acidosis and glucopenia, and following 1 h of reperfusion with normal Kreb's solution. The specimens were processed for immunofluorescence and for routine haematoxylin and eosin (H&E) staining.[17] For immunofluorescence, after fixation in 4% paraformaldehyde, the tissues were transferred into 30% sucrose in phosphate buffer and kept at 4°C overnight. The samples were then frozen in OCT compound (BDH, Poole, UK) and serial cryosections at 20 μm interval were obtained using a cryostat (18°C; 2800 Frigocut-E; Leica, Bensheim, Germany). The sections were dried on gelatine-coated slides for 2 h at room temperature and then incubated with phosphate-buffered saline (PBS) containing 0.1% Triton X-100 and 5% goat serum (Vector, Burlingame, California, USA). The slides were incubated with TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) reaction mixture (Roche, Lewes, UK) overnight at 37°C and then with a monoclonal antibody against smooth muscle-specific α-actin (1/250; Sigma, UK) for 2 h at room temperature followed with fluorescein-conjugated anti-mouse IgG (1/100; raised in goat; Chemicon, Harrow, UK) for 1 h at room temperature. The images were obtained using a laser-scanning confocal microscope (Leica TCS-DMRE, Germany). Image analysis was performed using Leica Confocal Software (Version 2.00, build0871, Germany). In order to avoid day-to-day variation in signal intensity, several sections from different experimental groups were immunostained and analysed in the same batch on the same day. The laser intensity and gain functions were set according to the control tissue; thereafter, these settings were applied to all sections from all experimental groups within the same batch. The results from TUNEL staining were expressed as the percentage of smooth muscle cells with TUNEL-positive nuclei.

Chemicals and solutions

All chemicals were obtained from Sigma (UK) unless otherwise stated. The Kreb's solution comprised (mM) NaCl 133, KCl 4.7, NaH_2PO_4 16.3, $NaHCO_3$ 16.3, $MgSO_4$ 0.61, $CaCl_2$ 2, glucose 7.8 and dexamethasone 0.01. The inducible form of NOS (iNOS) can be induced by trace amounts of endotoxin in the buffer[18] and cause loss of tone.[19] Dexamethasone was added to the Krebs' solution to prevent induction of iNOS.[18]

Statistical analysis

All results were expressed as mean ± s.e.m. Isometric tension was expressed as a percentage of the initial tone. Statistical analysis for pharmacology experiments was performed using Student's unpaired *t*-test. For the ATP measurements, Wilcoxon's signed-rank test for paired samples was used where the tissues from the same animals were paired. *P*-value less than 0.05 was considered significant. The statistical analysis was performed using GraphPad Prism software (version 3.00, Graphpad Software Inc, San Diego, California, USA).

Results

Protocol 1—Acute effect of hypoxia, acidosis and glucopenia on phenylephrine-induced tone: The constituents of the Kreb's solution were altered such that each parameter was investigated either individually or in combination. Hypoxia, acidosis or glucopenia alone reduced the tone of precontracted strips to 28.4 ± 2.9, 59.6 ± 7.5 and 9.4 ± 3.8% of the initial tone, respectively. Combinations of glucopenia + acidosis, glucopenia + hypoxia and hypoxia + acidosis reduced the tone to 13.9 ± 2.8, 8.1 ± 2.5 and 6.4 ± 3.1% of the initial tone, respectively. The combination of hypoxia, acidosis and glucopenia resulted in a loss of tone that was 8.2 ± 1.6% of the initial tone. These results are summarised in Figure 1. The time taken to reach the minimum tone is shown in Table 1 for each condition. Glucopenia resulted in the slowest rate of loss of tone.

Protocol 2—Time course for the recovery of phenylephrine-induced tone after reversal of combination of hypoxia, acidosis and glucopenia: The control tissue lost some of its tone during 6 h. The combination of hypoxia, acidosis and glucopenia resulted in a loss of tone, which progressively became irreversible after correction of the conditions. A sudden drop in the recovered tone was observed between 1 and 2 h (Figure 2). Since the maximum irreversibility was reached at 4 h, this time point was used in further experiments.

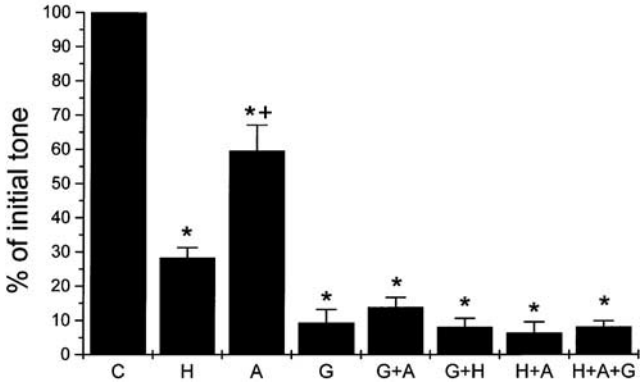


Figure 1 Acute effect of hypoxia (H), acidosis (A), glucopenia (G) and their combinations to phenylephrine-induced tone of the rabbit corpus cavernosum. Control (C) tissue was superfused with normal Kreb's solution. The tone was measured when the tissue reached a plateau. Each data point represents mean ± s.e.m. of four to 10 separate tissue strips from different animals. $^{*}P<0.05$ significantly different from control; $^{+}P<0.05$ significantly different from other treatments.

Protocol 3—Recovery of phenylephrine-induced tone after reversal of hypoxia, acidosis and glucopenia: At the end of 4 h, by reintroducing each parameter, the recovery of smooth muscle tone was recorded. Expressed as a percentage of the initial tone, the results showed almost complete recovery following the reversal of all the parameters except when glucopenia was combined with hypoxia or in the presence of combination of hypoxia, glucopenia and acidosis (Figures 3 and 4). The time taken to recover and to reach a plateau is summarised in Table 1 for each condition. The fastest recovery was observed when acidosis was used alone.

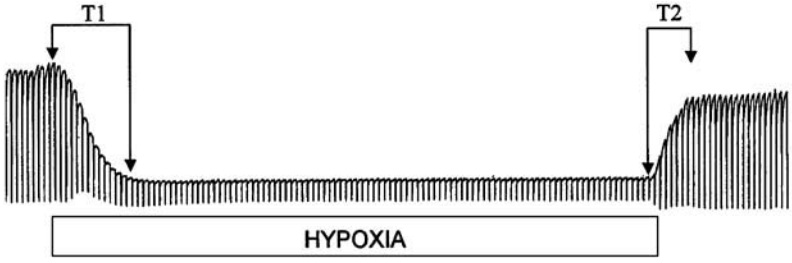
Protocol 4—Cumulative concentration–response to phenylephrine with or without hypoxia, acidosis and glucopenia: Cumulative addition of phenylephrine (0.1–300 μM) to tissue strips after superfusion with normal Kreb's solution resulted in a contraction with $EC_{50} \sim 10\,\mu M$ and a maximum tone of 3.1 ± 0. 7 g. After superfusion with a combination of hypoxia, acidosis and glucopenia for 4 h, and then superfusion with normal Kreb's solution, phenylephrine concentration–response curve gave an $EC_{50} \sim 3\,\mu M$ ($P<0.05$ *vs* control) and a maximum tone of 0.9 ± 0.2 g ($P<0.05$ *vs* control) (Figure 5).

Protocol 5—Effect of hypoxia, acidosis and glucopenia on nitrergic responses: EFS evoked reproducible and frequency-dependent relaxation responses after inhibition of noradrenergic and cholinergic pathways and elevation of the tone. These EFS-induced relaxation responses were completely inhibited with L-NAME (500 μM) or TTX (3 μM) (not shown), confirming that they are nitrergic in nature

Table 1 Time in minutes to reach the minimum recorded tone upon exposure to different conditions (*T1*) and to recover (to reach a plateau) after 4 h of exposure (*T2*)

Condition	T1	T2	n
Hypoxia	29.1 ± 2.2	20.8 ± 1.5	6
Acidosis	13.7 ± 1.1	11.5 ± 1.0**	4
Glucopenia	74.1 ± 15.0*	31.1 ± 4.1	5
Glucopenia + acidosis	70.4 ± 7.1	22.9 ± 1.1	5
Glucopenia + hypoxia	45.6 ± 4.4	32.7 ± 3.3	5
Hypoxia + acidosis	52.7 ± 4.6	42.1 ± 4.8	5
Hypoxia + acidosis + glucopenia	39.1 ± 1.9	24.1 ± 2.5	10

Measurement of *T1* and *T2* are explained below in a sample tracing. The results are expressed as mean ± s.e.m. *$P<0.05$ significantly different from hypoxia *T1* or acidosis *T1*.
**$P<0.05$ significantly different from hypoxia *T2* or glucopenia *T2*.
T1 = time to reach minimum recorded tone.
T2 = time to recovery.



and neuronal in origin. Incubation (4 h) with a combination of hypoxia, acidosis and glucopenia reduced the tone of the strips as mentioned above. During this period, nitrergic responses remained unchanged when expressed as the percentage of the tone (Figures 3c and 6). Following the reversal of hypoxia, acidosis or glucopenia, the tone partially recovered as shown in Figures 3 and 4 and nitrergic relaxations appeared to be preserved (Figures 3c).

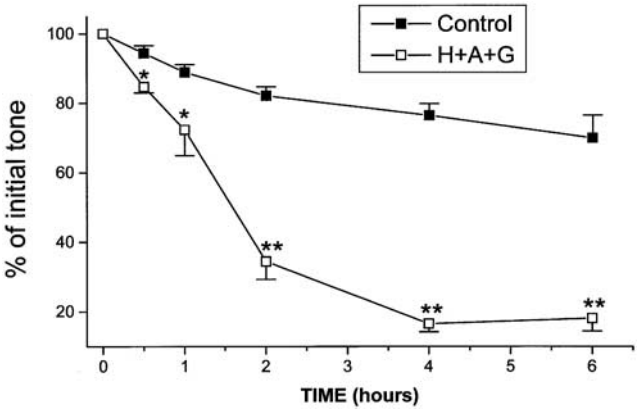


Figure 2 Time course for the recovery of phenylephrine-induced tone after the reversal of combination of hypoxia, acidosis and glucopenia (H + A + G; open squares). The control tissue (Control; solid squares) lost some of its tone during 6 h. The loss of tone became progressively irreversible after exposure to H + A + G reaching a maximum at 4 h. The tone was measured when the tissue reached a plateau after the reversal of the conditions. Each data point represents mean ± s.e.m. of four to 10 separate tissue strips from different animals. *$P<0.05$; **$P<0.0001$ significantly different from control at the same time point.

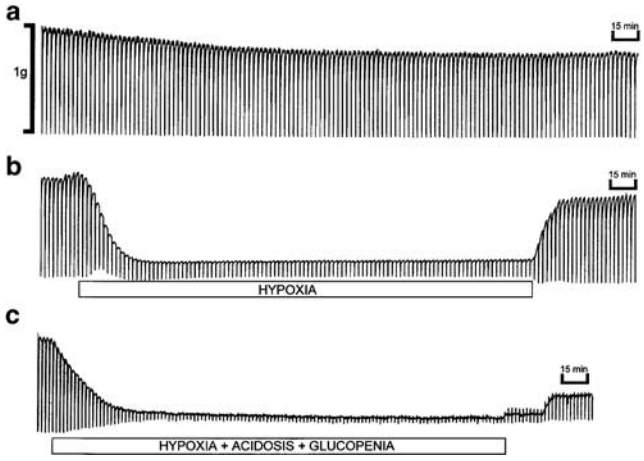


Figure 3 Original tracings from three cavernosal strips in the presence of normal (a), hypoxia (b) and the combination of hypoxia, acidosis and glucopenia (c) after treatment with scopolamine, guanethidine and phenylephrine. EFS (50 V, 0.3 ms pulse duration, 5 Hz) was applied every 2 min, which produced reproducible nitrergic relaxation responses. Note that the tone fails to rise after cessation of 4 h of hypoxia + acidosis + glucopenia.

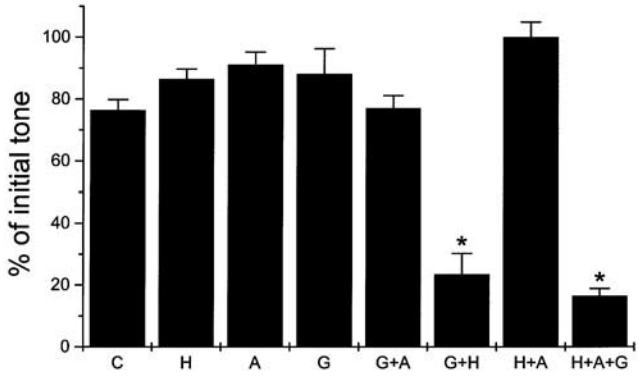


Figure 4 Recovery of phenylephrine-induced tone after reversal of hypoxia (H), acidosis (A), glucopenia (G) or their combinations after 4 h. The control tissue (C) lost some of its tone during this time period. The loss of tone was irreversible only in G + H and H + A + G groups. The tone was measured when the tissue reached a plateau after reversal of the conditions. Each data point represents mean ± s.e.m. of four to 10 separate tissue strips from different animals. *$P<0.05$ significantly different from control.

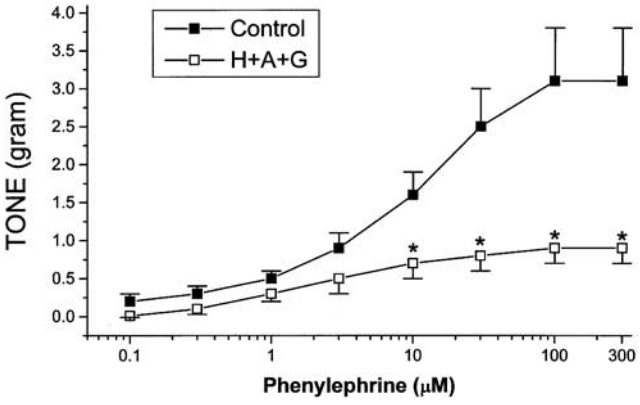


Figure 5 Phenylephrine concentration–response curves in control tissues, which were superfused with normal Kreb's solution throughout the experiment (Control; solid squares) and in tissues that were exposed to the combination of hypoxia, acidosis and glucopenia (H + A + G; open squares) for 4 h and then superfused with normal Kreb's solution. Each data point represents mean ± s.e.m. of four to six separate tissue strips from different animals. *$P<0.05$ significantly different from control at the same concentration of phenylephrine.

Concentration of ATP in tissue strips

ATP levels were measured at four time points: following the equilibration period, following precontraction with phenylephrine, following 4 h superfusion with a combination of hypoxia, acidosis and glucopenia, and following 1 h reperfusion in the normal Kreb's solution. ATP concentrations at those time points were measured as 60.2 ± 9.3, 65.3 ± 5.2, 46.3 ± 4.3* and 48.7 ± 2.4* pmol/μg protein, respectively (*$P<0.05$ significantly different from the first two time points using Wilcoxon's signed-rank test for paired samples). These results indicate a significant fall in tissue ATP content following 4 h incubation with a combination of hypoxia, acidosis and glucopenia. On reintroduction of oxygen, normal pH and glucose, the tissue ATP content failed to return to basal levels.

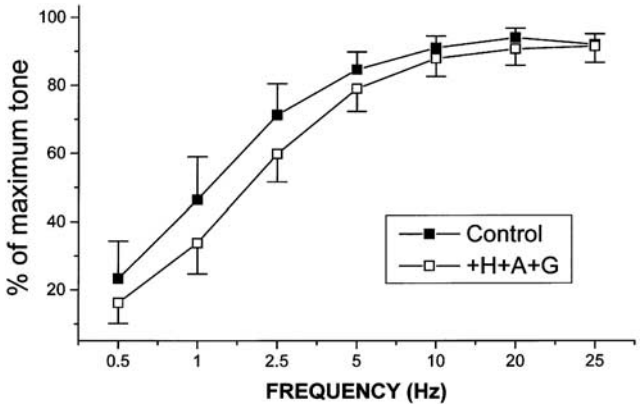


Figure 6 Nitrergic relaxations were not affected by hypoxia, acidosis and glucopenia. Frequency–response curves of nitrergic relaxations expressed as the percentage of the maximum tone in control tissues, which were superfused with normal Kreb's solution throughout the experiment (Control; solid squares) and in tissues that were exposed to the combination of hypoxia, acidosis and glucopenia (H + A + G; open squares) for 4 h and then superfused with normal Kreb's solution. Each data point represents mean ± s.e.m. of four to six separate tissue strips from different animals.

Histological changes in the rabbit corpus cavernosum

Histological analysis of the strips using H&E staining showed no significant change in the smooth muscle and endothelial structures after incubation with a combination of hypoxia, acidosis and glucopenia for 4 h (Figure 7a). However, immunofluorescence studies showed a significant increase in the number of TUNEL-positive nuclei in the smooth muscle cells under these conditions. Reperfusion with normal Kreb's solution failed to reverse the increase in TUNEL staining. Hypoxia alone caused an increase in TUNEL staining which however was not as much as the combination of hypoxia, acidosis and glucopenia (Figure 7b). Under control conditions less than 1% of smooth muscle cells were TUNEL positive. TUNEL-positive smooth muscle cells were 66.5 ± 2.5% of the total smooth muscle cells after 4 h of hypoxia ($P<0.05$ *vs* control). After 4 h of a combination of hypoxia, acidosis and glucopenia, this percentage was 88.6 ± 6.9% ($P<0.05$ *vs* control and *vs* hypoxia). Reperfusion of the tissue with normal Kreb's solution for 1 h after 4 h of hypoxia, acidosis and glucopenia did not alter the TUNEL staining (not shown).

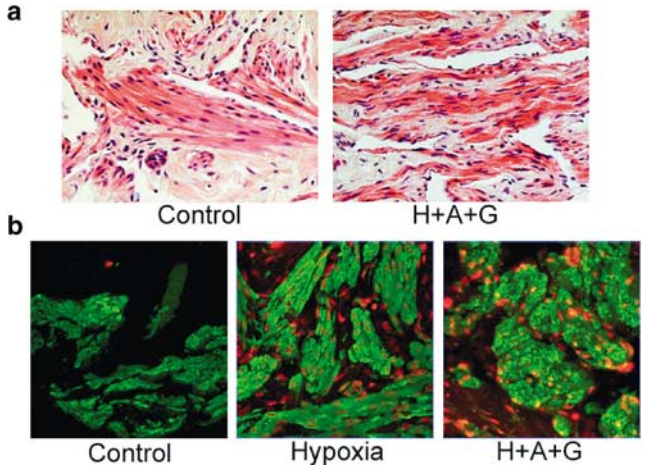


Figure 7 (a) Ischaemia for 4 h does not cause morphological changes. H&E staining of sections from two strips of the same corpus cavernosum that were exposed to normal Kreb's solution (Control) or to the combination of hypoxia, acidosis and glucopenia (H + A + G) for 4 h. (b) TUNEL staining increases with hypoxia or hypoxia, acidosis and glucopenia (H + A + G) in combination in cavernosal smooth muscle cells. Smooth muscle-specific α-actin (green) and TUNEL-positive cell nuclei (red).

Discussion

In order to achieve penile detumescence smooth muscle contractile mechanisms adequately (ie α-adrenergic stimulation, endothelin and myogenic tone), it must overcome the ones that cause relaxation (ie nitric oxide, prostaglandins). Although the degree of oxygenation, acidosis and substrate depletion can alter the contractile activity of the cavernosal smooth muscle, it does not necessarily cause irreversible smooth muscle dysfunction. Therefore we studied the effect of hypoxia, acidosis and glucopenia on the smooth muscle tone as well as on the capacity of the tissue to contract once the conditions were returned to normal.

Our study shows that in the presence of hypoxia alone, the tone of the cavernosal smooth muscle is decreased in accordance with previous studies.[4,5] During 4 h of hypoxia, the tone remained low. Once the tissue was reoxygenated, it regained its original tone, suggesting that hypoxia alone does not result in irreversible smooth muscle dysfunction. The reversibility of smooth muscle tone following reoxygenation has been reported in the corpus cavernosum in a previous study, although this was following a shorter period of hypoxia.[5]

The contraction of cavernosal smooth muscle depends on the mobilisation of Ca^{2+} from the sarcoplasmic reticulum and also on the transmembrane Ca^{2+} influx. Kim *et al*[5] showed that intracellular Ca^{2+} levels increase during exposure to hypoxia. Hypoxia-induced loss of tone in previous studies[4,5] suggests that either the sensitivity of the contractile apparatus to intracellular Ca^{2+} is altered during hypoxia[20] or the contractile apparatus becomes unresponsive to increased Ca^{2+} due to ATP depletion.[5] Our findings showing that hypoxia-induced loss of tone was reversible suggests that whatever the mechanism, it is more likely to be a reversible alteration either in Ca^{2+} sensitivity or ATP metabolism.

Acidosis has been suggested to be involved in impaired contractility during priapism.[6,10] We have shown in this study that acidosis alone causes loss of tone. Once the pH was reverted back to 7.4, the tissue regained most of its tone, suggesting that acidosis on its own does not cause irreversible dysfunction in the corpus cavernosum. To our knowledge, this is the first study to demonstrate the recovery of the tone after reversing the acidosis.

In our study, the pH of the Kreb's solution was lowered from 7.4 to 6.9 by reducing the bicarbonate content, a method known to reduce the extracellular pH. Reducing extracellular pH has been shown to decrease intracellular pH.[21] In a previous study, acidosis was simulated by gassing the solution with 75% O_2 + 25% CO_2, which confers intracellular acidosis.[6] This suggests that a reduction of intracellular pH by either method produces a sustained reduction in smooth muscle tone.

It has been shown that the reduction in the tone of the rabbit corpus cavernosum in acidotic conditions is associated with a concomitant decrease in intracellular Ca^{2+} concentrations.[6] Interestingly, acidosis resulted in the most rapid loss of tone in comparison to hypoxia or glucopenia. This suggests that acidosis might be exerting its depressant effect on the cavernosal smooth muscle through a different mechanism than hypoxia. In our study after 4 h of a combination of hypoxia and acidosis, the tone of the cavernosal smooth muscle completely reversed to its initial level, suggesting that the effect of hypoxia and acidosis on the contractile mechanisms is reversible.

We have shown in this study that withdrawal of glucose from the Kreb's solution results in a loss of tone, which was reversed when the glucose was replenished after 4 h. To our knowledge, this is the first study that showed the effect of glucopenia alone on the cavernosal smooth muscle. Glucopenia on its own has been shown to impair contractile responses in guinea-pig intestine[13] and detrusor muscle,[14] which recovered after the reintroduction of glucose.[13] Taken together, glucopenia on its own is unlikely to cause irreversible smooth muscle dysfunction.

A previous study has shown that the combination of hypoxia and glucopenia for 1 h resulted in a significant but reversible loss of tone in the rabbit corpus cavernosum.[8] In our study, the tissue failed to regain its tone after 4 h incubation with a combination of hypoxia and glucopenia, suggesting that irreversible smooth muscle dysfunction is time dependent and is more likely to occur in periods more than 2 h as suggested in our time-course experiment. The irreversible smooth muscle dysfunction as a result of combination of hypoxia,

acidosis and glucopenia was progressive and time dependent with a sudden drop after the 1 h time point. This is most probably due to *de novo* expression of proteins, which are involved in smooth muscle dysfunction.

Smooth muscle requires an adequate supply of ATP to maintain its contractile activity. Oxidative phosphorylation and glycolysis supply ATP under aerobic conditions. Under anaerobic conditions, glycolysis becomes the sole metabolic pathway through which ATP can be produced. In the absence of glucose, intracellular glycogen stores act as a source of glucose. In a condition where anoxia and glucopenia are combined, one would expect to see a gradual decrease in ATP levels leading to eventual ATP depletion. The rate of ATP decline would depend on the rate of glycogen breakdown and on the intensity of the glycogen stores. In our experiments, tissue ATP levels were significantly decreased following the introduction of hypoxia, acidosis and glucopenia combined. These ATP levels were unable to be replenished following reperfusion with normal Kreb's solution, suggesting that irreversible smooth muscle dysfunction could be partly due to the decreased ATP levels in the smooth muscle.

We have incubated the tissues in the ischaemic conditions (hypoxia, acidosis and glucopenia) for 4 h before measuring the ATP levels. Although the decrease in ATP levels at the end of this period was significant, it was not completely depleted. This might be due to some level of residual oxidative phosphorylation, since our conditions were not completely anoxic or due to some glycogen stores left in our preparations fuelling glucose into glycolytic ATP production. Further experiments measuring glycogen in totally anoxic conditions are required to confirm this.

Glucopenia alone induced slowest rate of tone loss in comparison to hypoxia and acidosis. This further suggests that there are likely to be sufficient glycogen stores in the cavernosal smooth muscle that are supplying glucose in the absence of extracellular glucose.

TUNEL staining detects DNA fragmentation, which is generally considered to be specific to apoptotic cell death, although necrosis has been reported to cause 'nonapoptotic' DNA fragmentation.[22] Therefore, we used TUNEL staining to detect apoptotic or necrotic cell death. Morphologically H&E staining did not reveal any changes after 4 h of hypoxia, acidosis and glucopenia, although cell death was evident using TUNEL staining. This suggests that classical features of cell death, such as shrunken cytoplasm and nuclear alterations that follow DNA fragmentation, have not started in our samples.

In our experiments, smooth muscle cell death occurred in the presence of hypoxia, glucopenia and acidosis, although the ATP levels were not completely depleted and the level of oxygen tension was not as low as anoxia. This suggests that other factors such as hypoxia-inducible factor[23,24] or anti-apoptotic protein[25] might be involved in the initiation of ischaemic cell death before ATP depletion occurs. Investigation of these pathways in priapism is currently in progress in our laboratory.

Interestingly, the EFS-induced nitrergic responses appear to be preserved during and after the combination of hypoxia, acidosis and glucopenia, although it was very difficult to assess the nitrergic relaxations at such low tone. This would suggest that the axonal terminals within the corpus cavernosum remain functional despite low levels of ATP. In contrast in the bladder detrusor muscle, the neural pathways are impaired before smooth muscle contraction during ischaemia.[14] Nitrergic axons as well as endothelial cells (as other source of NO) in the corpus cavernosum need to be analysed morphologically in order to clarify whether they are more resistant to ischaemia than the smooth muscle cells.

In order to ascertain that the loss of tone during hypoxia, acidosis and glucopenia in our experiments was not due to desensitisation of adrenergic receptors, we have repeated the experiments using endothelin-1 or U-46619 (thromboxane A_2 analogue) where similar results were obtained (unpublished observations). This indicates that the impaired recovery of smooth muscle tone is not exclusively a phenylephrine/α receptor-mediated event.

We have also repeated the same series of experiments in the presence of an inhibitor of NO synthase (L-NAME) where no change in reversibility after 4 h of hypoxia, acidosis and glucopenia was observed (unpublished observations). Although NO-mediated smooth muscle relaxation is by far the most important pathway mediating penile erection, our results indicate that smooth muscle relaxation due to ischaemia is not mediated by NO.

Taken altogether, we suggest a sequence of events that can help in our understanding of the changes occurring in penile tissue during prolonged ischaemic priapism (Figure 8). Following the onset of priapism, the corpus cavernosum acts as a closed compartment, which initially becomes hypoxic and develops a degree of acidosis. At this stage, reversing the hypoxia and acidosis using aspiration and/or drainage followed by reperfusion with normoxic blood is likely to allow complete smooth muscle recovery. At this stage, administration of α-agonists is unlikely to produce detumescence unless preceded with aspiration, drainage and reperfusion. We call this period 'window of opportunity' to highlight the importance of aspiration and reinstatement of reperfusion for the recovery of smooth muscle function. With prolonged ischaemic priapism, glucopenia develops as well as hypoxia and acidosis. Once all three factors are combined, ATP levels are irreversibly decreased in the smooth muscle cells and (apoptotic) cell death is initiated. This results in irreversible smooth muscle dysfunction. In a clinical setting, if after aspiration,

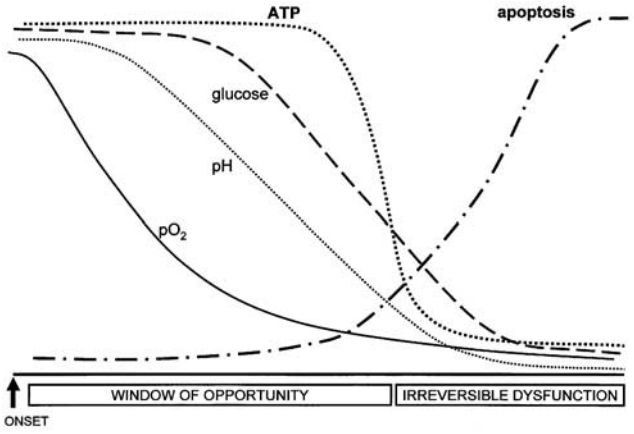


Figure 8 Hypothetical schematic presentation of the events in low flow priapism. After the onset, firstly oxygen concentration falls followed by a decrease in pH and then a decrease in glucose concentration. This hypoxic, acidotic and glucopenic microenvironment leads to a decrease in intracellular ATP concentrations and to the apoptotic process. This results in smooth muscle cell loss. If aspiration, reperfusion and α-agonist administration are performed before the ATP depletion, tumescence can be achieved within the 'window of opportunity'. Once this window is missed, smooth muscle dysfunction becomes irreversible.

reperfusion and instillation of α-agonists, there is still a failure of detumescence, this would indicate irreversible smooth muscle dysfunction due to smooth muscle cell death. Eventually, inflammatory cell infiltration followed by fibroblast proliferation accounts for the development of penile fibrosis, which is frequently observed in ischaemic priapism cases.[26]

In conclusion, our study suggests that combination of hypoxia, acidosis and glucopenia in low flow priapism results in decreased ATP concentrations and cell death, which leads to irreversible smooth muscle dysfunction in the corpus cavernosum.

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