Work in progress

Characterization of early thermal burns and the effects of hyperbaric oxygen treatment: a pilot study

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Abstract

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Background and aims: Studies investigating hyperbaric oxygen treatment (HBOT) to improve outcome in burns have been inconclusive. In this study, we aimed to characterize early thermal burns injury in adult patients with < 40% total body surface area (TBSA) and to determine the effects of HBOT administered within 24 h to 48 h of a burn injury.

Methods: Seventeen subjects were randomized into control ($n = 9$) and HBOT treatment ($n = 8$) arms. Burn depth, measured by laser Doppler imaging (LDI) and histologically, white blood cell (WBC) count and plasma cytokine inflammatory markers were assessed at 24 h (pre HBOT) and 48 h (post HBOT) post burn, as were immunohistochemistry and microbiology of burns tissue samples at 48 h post burn.

Results: WBC count and serum interleukin (IL)-1β, IL-4, IL-6, IL-10 and interferon-γ were significantly elevated 24 h after burn, but no significant changes in any of these parameters were found with HBOT. HBOT had no significant effect on burn depth. Two HBOT patients and four control patients developed positive bacterial cultures.

Conclusions: Slower than anticipated recruitment resulted in considerably fewer patients than planned being studied. Inflammatory markers were significantly increased at 24 h in patients with < 40% TBSA burn. Early HBOT had no apparent effects on any of the parameters measured in this small pilot study. HBOT may possibly have a broad-spectrum antimicrobial effect worthy of further study. We report our methodology in detail as a possible model for future burns studies.

Key words

Burns, hyperbaric oxygen therapy, Doppler, inflammation, bacteriology

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Introduction

Burn injuries continue to result in long-term morbidity and mortality.[1] The depth of burns is not static and parts of the burn wound may 'convert' (progress to become deeper) over the first three to five days.[2] Three concentric zones of burn injury have been described: irreversibly injured tissue in the zone of coagulation, hypoperfused tissue in the zone of stasis and oedematous tissue in the zone of hyperaemia.[3] Although the zone of stasis is potentially salvageable, it is at risk of necrosis in the event of suboptimal treatment.[4] Burns wound conversion is clinically important but poorly understood.[5] Burns conversion directly affects morbidity and mortality since, as the extent of the burn increases, there is a greater need for burns excision, as well as more hypertrophic infections, sepsis, scarring, contracture and mortality.[6] Hence, a reduction in burns conversion should improve clinical outcomes and length of stay for burns patients. The mainstay of modern burns care currently involves a multidisciplinary approach, including urgent fluid resuscitation, early intensive care, early excision and coverage of the burn wound within the first three to five days.[7,8]

Hyperbaric oxygen therapy (HBOT) has been identified as a possible adjunct to burns management.[9,10] Burns have been associated with impaired microcirculation.[11] It has been reported that hyperbaric hyperoxia reduces microthrombi formation through the inhibition of leukocyte adhesion by inhibiting the activation of intracellular adhesion molecule-1, allowing the maintenance of the microvasculature and prevention of reperfusion injury.[11,12] A Cochrane review recommended that more reliable clinical data from large, randomised controlled trials (RCTs) was required before HBOT could be considered a routine treatment for thermal burns.[13] To our knowledge, there has been no RCT that studies the role of HBOT as an adjunct to modern burns care. Our group aimed to conduct a pilot RCT of HBOT in that context. Our hypothesis was that HBOT would reduce burns conversion through the mechanisms summarised above, the aim being to complete two HBOT sessions prior to early excision on day three post burn. To better understand the mechanism of action of HBOT, we also proposed to measure changes in systemic immunological markers and the immunohistochemistry and microbiology of the burn injury.

Materials and methods

STUDY DESIGN AND PATIENT RECRUITMENT

The study was an un-blinded, prospective randomized trial comparing standard modern burns wound care with the same level of care combined with HBOT given in the first 48 h post burn. The study was approved by the Singapore

General Hospital (SGH) Institutional Review Board (IRB) and registered at: https://register.clinicaltrials.gov (NCT00824551) and conducted according to the principles of the Helsinki Declaration. Following evaluation and informed consent, the subjects were randomized in blocks of 10 using a fixed sequential list generated by computer and sealed in opaque envelopes. The inclusion criteria were:

- age 21–60 years;
- thermal burns injury covering < 40% of total body surface area (TBSA) with areas of deep dermal/full thickness burns;
- admission < 24 h from time of injury.

Patients who had any co-morbidities or required endotracheal intubation were excluded. Between 2008 and 2010, 110 burns patients were admitted to the SGH Burns Centre of whom only 18 met these strict criteria. All patients received standard burns management at SGH, including exposure to ambient room temperature of 22–30^{O}C, use of bio-occlusive dressings after thorough cleansing, adequate resuscitation using the Parklands formula, provision of blankets and the administration of adequate analgesia but excluding non-steroidal anti-inflammatory drugs.[14] Any patients febrile above 38^{O}C had a full sepsis work up.

HBOT INTERVENTION

The HBOT protocol was chosen based on safety, research aims and practicality. Patients were treated on the routine HBOT runs, and completed two sessions within 22 hours of admission. Each HBOT session consisted of 90 min at 243 kPa breathing 100% oxygen, with an ambient room temperature of 22–30^{O}C. The minimum interval between the two sessions was 120 minutes. Sham hyperbaric treatments for control subjects were not performed.

BLOOD PROCESSING

Blood samples (15 ml) were collected into four containers before and after HBOT and at similar times in the control group via needle-stick venepuncture; one was sent for routine laboratory analyses, including white blood cell (WBC) count and differential. Three plastic serum separator tubes, each containing approximately 3 ml whole blood, were inverted five times and allowed to clot for 30 minutes at room temperature. These were then centrifuged at 1,200 rpm for 15 minutes at 4^{O}C and stored at -80^{O}C until analysis. Serum interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p70), IL-13, interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α) levels were measured using a Bio-Plex system (Precision Pro Human Cytokine 10-plex panel, 171-A1001P, Bio-Rad Laboratories, Inc., USA). The choice of cytokines measured was based on the cytokines that had previously been reported to be elevated with burns.[15–17]

ASSESSMENT OF BURN DEPTH

Laser Doppler imaging

Laser Doppler imaging (LDI) was chosen as an objective, independent assessment tool.[12] A Moor Instruments LDI2-Burn Imager (BI) (Moor Instruments Ltd., Axminster, England, UK) was used. All patients underwent LDI scans before and after HBOT or at similar times in the control group. The LDI flux values were based on an improved colour palette used to interpret burn depth in previous studies: < 260 perfusion units (PU, unit for flux based on a range corresponding to the visible spectrum) for deep dermal and full thickness burns (blue and green); 260–800 PU for superficial dermal wounds (yellow, pink and red).[18] The areas of burns that fall into the pre-defined PU ranges can be marked out and measured using a touch screen on the LDI as regions of interest.

HISTOLOGICAL ASSESSMENT

A biopsy within the LDI-assessed deep dermal burn was taken from each patient 48 h post burn. Skin biopsy specimens were fixed by immersion in 4% formalin for 48 h and then dehydrated in alcohol, cleared with xylene, and embedded in paraffin wax. Sections of 4 μm thickness were cut and microwaved in citrate buffer for antigen retrieval and blocked with peroxidase blocking reagent (S2023, DAKO UK Ltd, UK). Sections were stained using hematoxylin and eosin (H&E) for general morphology. Skin samples were classified into five anatomical layers: epidermis (level 1), upper one-third of the dermis (level 2), middle third of the dermis (level 3), deepest third of the dermis (level 4) and subcutaneous fat (level 5). The epidermis was evaluated for burn artefacts (distortion of cell contour) and separation of epidermis from dermis (subepidermal blistering). The dermis was evaluated for the histological separation or destruction of different cell layers of hair follicles and vessel walls, microthrombi and infiltration of neutrophils. Burn depth at each evaluation was graded as 1–5 according to the depth of the deepest burn-related histological finding of each sample. In addition, sections were stained with Masson trichrome stain (HT15, Sigma-Aldrich), which stains denatured collagen red, to estimate full-thickness burn depth through disruption of the dermal layer.

Immunohistochemistry

For apoptosis staining, sections were stained according to the protocol provided in the ApopTag® Peroxidase in situ Apoptosis Detection Kit (S7100, Chemicon International, Inc MA, USA). For immunohistochemistry, sections were also incubated with monoclonal rabbit anti-CD11a (Ab52895, Abcam Inc MA, USA), diluted 1:100 in PBS; monoclonal mouse anti-CD68 (M0814, DAKO UK Ltd) diluted 1:150 in PBS; and polyclonal rabbit anti-vascular endothelial growth factor (VEGF) (Thermo Scientific, USA) diluted 1:100 in PBS for the detection of CD11a, CD68 and VEGF respectively. Subsequent antibody detection

Table 1

Summary of patient demographics, burn aetiology and total body surface area (TBSA) and treatment arm (n = 17); LDI – Laser Doppler imaging assessment of burn depth and burns conversion at 24 h and 48 h (n = 13), S - superficial (mean PU: 260 – 800), D – deep (mean PU: 0 – 260); H&E burn depth assessment at 48 h post-burn skin tissue (n = 15); M - male, F – female; N/A – not available

Age	Gender	Burn cause	TBSA (%)	LDI (24 h)	LDI (48 h)	H&E Score (48 h)
No HBO treatment						
24	M	Contact	2.5	N/A	N/A	N/A
24	M	Flash	17	N/A	N/A	4
28	M	Flame	35	N/A	N/A	3
59	F	Scald	3	S	S	0
38	M	Scald	7	D	D	4
47	F	Scald	8	D	D	4
30	M	Chemical	7	D	D	1
31	M	Scald	25	D	D	3
48	M	Scald	6.5	S	S	3
HBO treatment						
37	F	Flame	17.5	D	S	N/A
46	M	Scald	6.5	D	D	4
49	M	Flame	9.5	D	D	4
25	M	Scald	18	S	S	3
55	M	Flame	18	N/A	N/A	4
48	F	Scald	8	D	D	4
25	F	Flash	1	D	S	4
29	M	Flame	3	S	D	3

was carried out using either anti-mouse or anti-rabbit IgG (Envision + system-HRP, DAKO UK Ltd) and then visualised using Vector® VIP Peroxidase Substrate (SK4600, Vector Laboratories, Inc., USA). All samples were examined under light microscopy. The number of immuno-positive cells and apoptotic cells were scored semi-quantitatively by an independent observer who was blinded to allocation.

MICROBIOLOGY

Quantitative culture and histological identification of bacteria in tissue specimens of viable unburned tissue has long been considered the gold standard for determining burns wound infection.[13–14] Tissue biopsies were taken both from areas of deep dermal burn and likely viable margins from each subject 48 h after burning. All positive cultures were quantified based on counts per gram of tissue and tested for sensitivity. Gram-negative isolates were tested for susceptibility to a range of antibiotics using the Kirby-Bauer disk diffusion method. Results of tissue cultures from the HBOT group were compared with those in the control group.

STATISTICS

Pre-trial power analysis based on an expected improvement of at least 10% in burn depth conversion in the HBOT group compared with the control group required a sample size of 40 subjects from each treatment group to achieve the limits defined ($\alpha = 0.05$; $\beta = 0.9$). Differences between the control and HBOT groups were compared using the Mann-Whitney U test when there was evidence of kurtosis/skew in the distribution of the values obtained. A one-sample Student's t-test was used to compare the burn levels against normal physiological levels (either the specified clinical range employed at SGH or, where no such values existed, derived from the blood of ten healthy control subjects). Pearson chi-square test was used to compare the incidences of positive microbiological tissue cultures. Data are expressed as means ± SD, where appropriate. Significance was accepted at a P value of less than 0.05.

Results

DEMOGRAPHICS

One patient was excluded because of newly discovered diabetes mellitus. Of the remaining 17 patients, 5 were female and 12 were male of varying burn aetiology, mainly from scalds (eight patients) and flame burns (five patients) (Table 1). Nine subjects (mean age 36.6, range 24–59 years) were assigned as controls and eight (mean age 39.0, range 25–55 years) to HBOT. The TBSA at admission in the control and HBOT arms were 13% (range 2.5–35%) and 12% (range 1–18%) respectively. There were no statistical differences between the two groups. The anatomic location of the injuries varied widely. No problems were encountered by the patients during HBOT.

WBC COUNT AND CYTOKINES

The WBC count was significantly raised immediately after burn compared with the maximum normal range (14 ± 5

Table 2
Systemic levels of haematological and cytokine markers of burn subjects at 24 h (n = 17); mean ± SD

Marker	Non-burn control	Burn	*P*- value
White blood cells (10^9 L^{-1})	4–10	14 ± 5	0.011
Neutrophil (%)	40–75	76 ± 12	0.73
IL-1β (pg ml^{-1})	0.12 ± 0.16	1.13 ± 1.24	0.004
IL-4 (pg ml^{-1})	0.02 ± 0.05	0.16 ± 0.23	0.023
IL-6 (pg ml^{-1})	0.73 ± 1.73	127.68 ± 174.66	0.012
IL-10 (pg ml^{-1})	0.28 ± 0.51	14.4 ± 20.42	0.014
IFN-γ (pg ml^{-1})	0.05 ± 0.15	0.74 ± 1.29	0.003

vs. 10 x 10^9 L^{-1}, P = 0.011), but there was no significant increase in neutrophils beyond the physiological range (76 ± 12% vs. 75%, P = 0.73). No significant changes in WBC count were found between the control and HBOT groups at either assessment time. Serum cytokines IL-1β, IL-4, IL-6, IL-10, and IFN-γ levels were significantly elevated after burn compared with non-burn control values, but there were no differences in systemic cytokine levels between the two patient groups (Table 2).

BURN DEPTH AND IMMUNOHISTOCHEMICAL CHANGES

Using LDI, 9 out of 13 subjects (LDI data were lost for four patients) were identified with deep dermal burns at 24 h whilst eight were identified with deep burns at 48 h. One HBOT subject had burns conversion from superficial to deep whilst one subject in the non-HBOT group and two in the HBOT group had conversion from deep to superficial burn (Table 1). There were no significant changes between the first and second assessments in either the control (pre 251 vs. post 271 PU, P = 0.522) or HBOT (pre 238 vs. post 244 PU, P = 0.949) groups. There were no significant differences between the two patient groups at either assessment (P = 0.475 and P = 0.253 respectively).

Two patients did not require skin excision surgery whilst, of the remaining 15, superficial to partial thickness dermal injury was observed in six. No distinction in histological observations between control and HBOT groups could be made based on burn depth scoring and collagen alteration. Comparison of H&E scoring with LDI assessment of burn depth at day two showed a discrepancy in only 2 out of 12 assessments (Table 1). TUNEL staining revealed apoptotic cells mainly in the upper half of the dermis, whilst CD11a and CD68 immunostaining showed varying degrees of leukocyte and macrophage infiltration in the burned skin sections respectively. VEGF immunostaining for mononuclear and polymorphonuclear leukocytes, endothelial cells and fibroblast-like cells also varied. No differences in immunostaining could be seen between the HBOT and control groups.

MICROBIOLOGY

Four subjects from the control group had positive bacteriological cultures versus two in the HBOT group. Several different organisms were identified, including *Staph. aureus* and *Pseud. aeruginosa*.

Discussion

We wished to assess the role of HBOT as an adjuvant to modern burns care. While our power calculation suggested we required 40 subjects in each arm of this study in order to confirm or refute a clinically significant effect of HBOT, a decrease from the predicted numbers of burns admissions in Singapore over the study period, combined with the strict entry criteria for this study, resulted in a very slow recruitment rate, preventing completion of the study to the level planned. Therefore, this trial can provide only a limited understanding of the effects of HBOT on early burns. However, we considered that it would be useful to describe our methodology in detail. To better understand the mechanism of action of HBOT on burns conversion, we measured an extensive range of biochemical and haematological indices (of which only WBC counts are reported here) and inflammatory cytokine markers. No significant differences were reported for any of these parameters following HBOT. Thus, we did not see any discernible effects of HBOT in this limited number of patients.

Our findings corroborate previous reports on the accuracy and reliability of LDI for assessing burn depth.[19] In this study, H&E tissue staining and LDI were in agreement in the assessment of burn depth in 10 of 12 patients for whom complete data were available (Table 1). We were unable to demonstrate whether there were any positive effects of HBOT on burns conversion, again, probably because of the low power of the study.

The lower proportion of patients with positive tissue cultures after HBOT suggests that HBOT might have a broad-spectrum antimicrobial action, but numbers were too small to

demonstrate any real differences. Nevertheless, this may be a useful area for future study given the known antimicrobial effects of HBOT.[20]

Conclusions

The WBC count was significantly elevated at 24 h after burn, along with systemic cytokines. We found no statistically significant differences between the control and HBOT groups in WBC count, inflammatory cytokine levels or microbiology. There was no evidence of improved burn conversion with HBOT. Unfortunately the study was of low statistical power because of recruitment problems and larger RCTs are still needed to determine whether HBOT has a place in modern burns therapy. LDI correlated well with H&E staining for flame and scald burns; LDI should be an effective independent assessment tool for burn depth and burn tissue for future research. Immunohistochemical evidence of macrophage infiltration and increased VEGF expression features strongly within 48 h of burn.

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Conflict of interest
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