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Effect of gamma-hydroxybutyrate on keratinocytes proliferation: A preliminary prospective controlled study in severe burn patients

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ABSTRACT

Background: Hypermetabolism and hyposomatotropism related to severe burns lead to impaired wound healing. Growth hormone (GH) boosts wound healing notably following stimulation of the production of insulin-like growth factor-1 (IGF1), a mitogen factor for keratinocytes. Gamma-hydroxybutyrate (GHB) stimulates endogenous GH secretion.

Aim: To assess effects of GHB sedation on keratinocytes proliferation (based on immunohistochemical techniques).

Design: Monocentric, prospective, controlled trial.

Materials and Methods: Patients (aging 18-65 years, burn surface area >30%, expected to be sedated for at least one month) were alternately allocated, at the 5th day following injury, in three groups according to the intravenous GHB dose administered for 21 days: Evening bolus of 50 mg/kg (Group B), continuous infusion at the rate of 10 mg/kg/h (Group C), or absence of GHB (Group P). They all received local standard cares. Immunohistochemistry (Ki67/MIB-1, Ulex europaeus agglutinin-1 and Mac 387 antibodies) was performed at D21 on adjacent unburned skin sample for assessing any keratinocyte activation. Serum IGF1 levels were measured at initiation and completion of the protocol.

Statistical Analysis: Categorical variables were compared with Chi-square test. Comparisons of medians were made using Kruskal-Wallis test. *Post hoc* analyses were performed using Mann-Whitney test with Bonferroni correction for multiple comparisons. A $P < 0.05$ was considered to be statistically significant.

Results: A total of 14 patients completed the study (Group B: $n = 5$, Group C: $n = 5$, Group P: $n = 4$). Continuous administration of GHB was associated with a significant higher Ki67 immunolabeling at D21 ($P = 0.049$) and with a significant higher increase in the IGF1 concentrations at D21 ($P = 0.024$). No adverse effects were disclosed.

Conclusions: Our preliminary data support a positive effect of GHB on keratinocyte proliferation and are encouraging enough to warrant large prospective studies.

Key Words: Burn injury, gamma-hydroxybutyrate, immunohistochemistry, keratinocyte

INTRODUCTION

Severe burn injury is associated to a hypermetabolic response, mediated by elevated catecholamines, corticosteroids, and proinflammatory cytokines. In addition, resistance to or suppression of circulating anabolic hormones is operative. Particularly, the growth hormone (GH)/insulin-like growth factor 1 (IGF1) axis is affected. This may lead to impaired wound healing. Pharmacological strategy aiming to modulate this severe and prolonged hypermetabolic and hypercatabolic response particularly includes recombinant human growth hormone (rhGH). Unlike general intensive care population,[1] no adverse impact on mortality was observed

in burn patients. Beneficial effects of rhGH on metabolic status and wound healing were demonstrated.[2] However, rhGH effects are not higher than oxandrolone (an alternative anabolic hormone),[3] while disclosing adverse hyperglycemia.[4] Unfortunately, drug costs limit its administration. This treatment is currently indicated only in severe burn of children,[5] for whom proved GH deficiency is associated with growth impair.[5]

Gamma-hydroxybutyrate (GHB) is a hypnotic drug inducing limited respiratory or hemodynamic depression.[6] Intravenous GHB is still used in clinical settings as an adjunct to anesthesia of hemodynamically instable patients[7] or sedation of intensive care patients.[8] Furthermore, GHB may have stimulatory effect on GH release.[9] Mechanism of this somatotropic action is not yet clearly understood: It could be related to its slow wave sleep induction. Indeed, there is a consistent relationship between the appearance of delta waves on the electroencephalogram (EEG) and GH secretion.[10]

To the best of our knowledge, only a single study has investigated the benefit of GHB administration on wound healing in burn subjects: It was an animal assay in a rat model.[11] As GHB can be considered as a GH-releasing agent, we hypothesized that GHB administered during the early hypermetabolic phase would stimulate proliferation of epidermal cells, through stimulation of the liver production of IGF1. The aim of the present preliminary human study was therefore to assess the effect of 21 days - GHB sedation on keratinocytes stimulation and proliferation, based on immunohistochemical techniques. Our secondary aim was to assess its effects on IGF1 blood concentrations.

MATERIALS AND METHODS

Subjects

The study protocol was approved by the local Ethics Committee of our Military Teaching Hospital and by the 8th Ethical Research Committee of the Ile de France Region (reference SC 10 06 60). The study was registered in the EudraCT database under reference 2010-A00475-34. Informed written consent was obtained from the family members of the patients prior to enrolment. The study was conducted from May 2010 to January 2011 in a 16-bed burn unit. Inclusion criteria were: Total burn surface area (TBSA) greater than 30%, expected sedation for at least one month, age between 18 and 65 years (delayed wound healing is observed after 65 years). Pregnancy, active cancer disease, pituitary or suprarenal insufficiency, diabetes mellitus, and chronic renal or liver failure were considered exclusion criteria.

Study protocol

Our study was a monocentric, prospective, controlled trial. All patients benefited from local standard monitoring and care procedures. Sedation was based on midazolam; analgesia was based on paracetamol and morphine. Enteral feeding was prescribed to obtain intakes of 35 kcal/kg/d and 0.4 g of nitrogen/kg/d. Diet was supplemented with ornithine α-ketoglutarate, a glutamine and arginine precursor, administered twice daily as an enteral bolus of 10 g (Cetornan®, Chiesi, France). Patients also received daily intravenous micronutrient supplementation: 1 g acid ascorbic, 100µg selenium, 5mg calcium folinate, a multivitamin preparation (Cernevit®, Baxter, USA), and a multi-trace-element preparation (Tracutil®, B Braun Medical, Germany). Intravenous insulin was administered to maintain blood glucose level between 0.8 and 1.4 g/l (euglycemia). Dextrose 5% or 2.5% (D5% or D2.5%) infusion rates were adjusted according to natremia. Hypokaliemia was treated using potassium chloride (KCl) supplementation. Deep burns benefited from early excision and grafting. Dressings were changed every third days under general anesthesia.

After inclusion, patients were assigned to receive intravenous GHB as 50 mg/kg bolus at 21 pm (*group B*) or as a continuous infusion (*group C*) at the rate of 10 mg/kg/h for 21 days, starting at the fifth day after injury (day 0 = D0). Patients assigned in *group P* did not receive GHB: They were considered as a negative control group. Allocation was based on admission timing: Patients were consecutively assigned in group C first, then in group B, and finally in group P. This pattern was repeated each 3 patients. Only intensivists and nurses were aware of this alternate allocation. Patients, surgeons, dermatopathologists, and clinical chemists were blinded to the group allocation.

There was no sample size calculation as it was a preliminary study with no relevant previous data. Number of admissions during the study period determined the sample size.

Evaluation of epithelial changes

Skin biopsy (4-millimeter punch) was performed on day 21 (D21) of the protocol in apparently healthy skin close to an unhealed burn area. Histopathology assessment were blinded when submitted to an experienced dermatopathologist. Microscopical sections (6-µm thick) were cut from the formalin-fixed paraffin-embedded punch biopsies. The sections were used for immunohistochemical assessments using a panel of antibodies (Ki67/MIB-1, 1:100, Dako®; UEA-1, 1:2000, Sigma® and Mac387, 1:200, Dako®) and the avidin-biotin peroxidase method. After a 1-h incubation time with any of the primary antibodies, the slides were washed in Tris-buffered saline (TBS) and incubated for 30 min with the secondary antibody (biotinylated swine antirabbit, 1:300, Dakopatts®). Slides were rinsed in TBS and covered by the EnVision (Dakopatts®,

Glostrup, Denmark) polymer-based revelation system. After TBS washings, Fast Red (Dakopatts®) was used as chromogen substrate. The last steps consisted of counterstaining with Mayer hemalum before mounting. Negative immunohistochemical controls were performed by omitting or substituting the primary and the secondary antibodies of the laboratory procedure.

Ki67 antigen is a nuclear protein expressed in all proliferating cells. Ki67/MIB-1 monoclonal antibody reveals the germinative compartment formed by all cells engaged in the cell cycle of proliferation. Mac 387 monoclonal antibody specifically identifies L1-protein. The L1-protein, corresponding to calprotectin, consists of three calcium noncovalently bound polypeptide chains. It is expressed in reactive epidermis but also in mucosal epithelium, neutrophils, monocytes, and some reactive macrophages. Ulex europaeus agglutinin-1 (UEA-1) reacts with fucose moieties present on cell-associated glycoproteins. It typically identifies reactive keratinocytes and endothelial cells.

The total number of Ki67+ keratinocyte was counted per mm length in 3 relevant contiguous sectors in each section before expressing these three counts as a mean number per biopsy specimen. The immunolabeling using UEA-1 and Mac 387 was assessed semi-quantitatively only in the epidermis in a 5-grade scale according to: 0 (absence), 1 (<1/3 epidermal thickness), 2 (>1/3 <2/3 epidermal thickness), 3 (>2/3 epidermal thickness with skipped areas), and 4 (full thickness).

Hormonal assessments

A blood sample, collected through a central venous line placed for clinical use, was obtained at 7 am of D0 and D21 for the measurement of IGF1. IGF1 levels were measured at that time of the morning for practical reason, as it is not release in a pulsatile way.

Blood was drawn into a serum gel tube (Vacutainer SST II Advance®, BD Diagnostics, USA), kept on melting ice, and quickly centrifuged (3500 rpm, 15 min, 4°C). Aliquots of the supernatant were finally stored at-80°C. Measurement of IGF1 concentrations was made within 3 months after the end of the protocol. Levels were measured using a commercially available immuno-luminometric assay (Liaison®, DiaSorin, Stillwater, MN, USA). Lower limit of sensitivity for IGF1 assays was 20 ng/ml.

Data analysis

The primary endpoint was the keratinocytes proliferation rate at D21, evaluated via the Ki67 immunolabeling. UEA-1 and Mac 387 labeling were also assessed in this aim. For each patient, results of the immunolabeling were considered as a mean of three sectors analyzed. The second endpoint was IGF1 concentrations. For each patient, these results were analyzed regarding the difference between values at D21 and D0 (delta IGF1).

Statistical analysis

Statistical analysis was performed using Stata 11.2 (StataCorp. 2009, College Station, TX, USA). Data are reported as ratio for proportions or as medians and ranges (min-max) for continuous variables. Categorical variables were compared with Chi-square test. Comparisons of medians were made using Kruskal-Wallis test. *Post hoc* analyses were performed using Mann-Whitney test with Bonferroni correction for multiple comparisons. A $P < 0.05$ was considered to be statistically significant.

RESULTS

A total of 14 patients were enrolled: They all completed the trial. Patients were all Caucasians. All cases were flame burns. Patient characteristics are presented in Table 1. Median age was 37 years old, median TBSA

Table 1: Patients characteristics

	Group B $n=5$	Group C $n=5$	Group P $n=4$	*P* value
Age (years)	38 (32-61)	33 (20-42)	45 (36-63)	0.11
Gender	2M/3F	5M	2M/2F	0.11
TBSA (%)	54 (27-61)	40 (35-65)	56 (39-72)	0.57
Deep BSA (%)	48 (14-55)	25 (23-55)	47 (17-50)	0.73
ABSI score	10 (9-12)	8 (6-9)	9 (6-14)	0.09
LOS (days)	52 (46-106)	47 (29-99)	71 (41-141)	0.45
Number of surgery	2 (1-4)	2 (1-3)	1 (0-3)	0.47
Delay between surgeries (days)	6.5 (6-18)	7.5 (4.6-17)	8.2 (5.5-11)	0.98
Excised surface area (%)	18 (6-41)	20.5 (3-39)	14 (0-36)	0.73
Proportion of healed burns (%)	66 (50-66)	70 (30-90)	52 (15-66)	0.35
PRBC (units)	13 (12-15)	11 (11-19)	12.5 (4-20)	0.63
Average Na (mmol/l)	142 (131-157)	140 (133-152)*	144 (135-156)	0,0001
Average K (mmol/l)	4 (3-4.7)	3.8 (2.9-4.7)*	4 (2.5-5.3)	0.0001
Insulin cumulated dose (UI)	170 (28-1224)	30 (0-316)	646 (114-2401)	0.2
Insulin (UI/kg/kcal/day)	0.2 (0-2.1)	0 (0-0.3)	0.9 (0.1-4.3)	0.14

TBSA: Total burn surface area, BSA: Burn surface area, M: Male, F: Female, LOS: Length of stay, PRBC: Packed red blood cells, Na: Blood sodium level, K: Blood potassium level. Data are presented as median (min-max) or proportions

reached 52%, and ABSI score was 9. No statistical differences on epidemiologic data were observed between the three groups. However, there were more men in group C than in the two other groups. Group C patients tended to suffer from more severe burns.

Despite the lack of significant difference in length of hospital stay between groups, it tended to be shorter in group C. Surgical strategy was similar among groups during the study period. Proportion of healed burns at the end of the study period tended to be higher in group C, but difference between groups was not statistically significant.

There were no statistical differences between the three groups regarding the number of packed red blood cells (PRBC) units delivered to patients between admission and the end of the study period. Only one patient in group B received fresh frozen plasma (3 units) during this time. No patient received platelet transfusion during the study period.

Despite the lack of statistical significance, patients of group C tended to receive less intravenous insulin than patients of group B and P.

At D21, the mean Ki67+ keratinocytes number was significantly different between the three groups ($P = 0.049$), respectively 11 (9-16), 23 (16-38) and 4 (1-20) in groups B, C and P [Figure 1]. After *post-hoc* analysis, there was a trend toward higher values in group C when compared with group B ($P = 0.07$). No difference between groups was observed when looking to UEA-1 or Mac 387 immunolabeling in the epidermis [Table 2].

Delta IGF1 was statistically different between the three groups ($P = 0.0264$): results in groups B, C, and P were respectively 58.4 ng/ml (12.06-100), 107.41 ng/ml (51.1-117.2), and 31.3 ng/ml (-2.29-40.9). *Post-hoc* analysis showed a significant increase of IGF1 concentrations in group C when compared with group P ($P = 0.042$) [Figure 2].

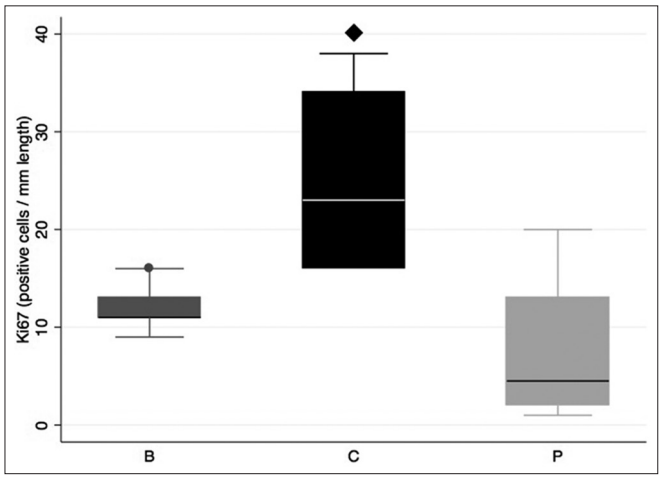

Figure 1: Ki67 labeling at D21 Graph shows Ki67 labeling at day 21 in each group. Black diamond indicates significant difference between the three groups ($P = 0.049$). *Post-hoc* analysis demonstrated a trend toward higher values in group C when compared with group B ($P = 0.07$)

No adverse events were disclosed under the present conditions of GHB administration. Patients of group B and C did not experience more severe hypernatremia or hypokaliemia than patients of group P [Table 1]. All included patients received similar amounts of free water infusion (D5% or D2.5%) and KCl.

DISCUSSION

Our preliminary data support a positive effect of GHB on keratinocyte proliferation. Such finding is in line with a previous report in a rat model.[11] Despite of the modest level of statistical significance in Ki67 immunolabeling (most probably due to the small number of subjects), continuous intravenous GHB infusion at the dose of 10mg/kg/h appeared to raise the keratinocyte proliferative rate in the skin adjacent to the burn area after 21 days of treatment. This finding can be considered relevant when referring to data previously outlined in a study aiming to describe the basic immunohistology of burn wound and adjacent unburned skin: An initial peak of Ki67 labeling was reported in both areas just after burn injury, followed by a marked labeling decrease after 20 days post-injury (number of positive cells being ≤5).[12] In the present study with continuous GHB infusion, the evolution pattern was the opposite: The number of positive cells remained quite much high. This could be explained by the mitogen effect of IGF1 on keratinocytes.[13] IGF1 secretion was possibly stimulated by the somatotropic effect of GHB. Indeed, we observed that IGF1 levels were on the rise after 21 days of continuous GHB infusion. In the present study, IGF1 secretion did not seem to be influenced by insulin: Patients of group C tented to receive lower intravenous insulin doses than the others.

A number of studies performed in healthy and narcoleptic subjects suggested that GHB represented the archetype of a novel class of GH secretagogue.[9,14] Administration of a modulator of endogenous GH secretion could be a novel approach in the management of burn injury. Effects of GH on wound healing are likely due to direct stimulation of cell division and protein synthesis.[15] Specific

Table 2: Immunohistochemical data at D21

	Group B $n = 5$	Group C $n = 5$	Group P $n = 4$	P value
UEA-1	1 (1-1)	1 (1-0)	1 (1-1)	0.51
Mac 387	2 (0-4)	3 (1-4)	3 (2-4)	0.30

UEA-1 and Mac 387: Semi-quantitative evaluation using a 5-grade scale. Data are presented as median (min-max)

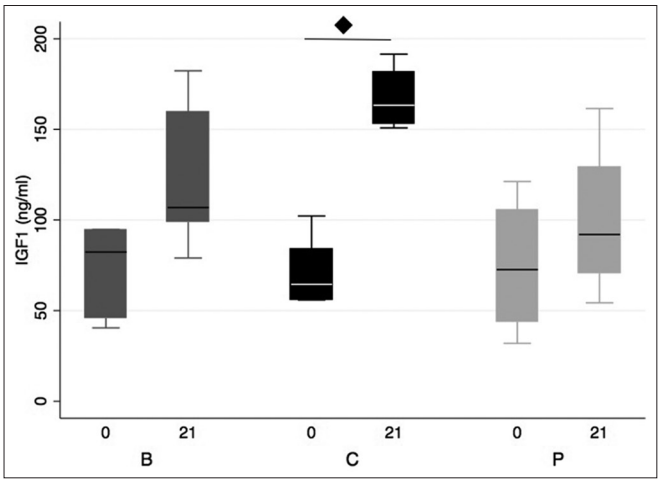

Figure 2: IGF1 blood concentrations at D0 and D21, over groups graph shows IGF1 blood concentrations at baseline (0 on x-axis) and at day 21 (21 on x-axis) for each group. Black diamond indicates a significant increase of IGF1 concentrations in group C when compared with group P ($P = 0.042$)

hormonal receptors are present on dermal fibroblasts and keratinocytes.[16,17] In addition, GH acts through stimulating IGF1 production by the liver.[18] IGF1 is known to be a mitogen for keratinocytes.[13]

Mechanism of GHB action is not yet fully understood. GHB is endogenously present in several tissues, but GHB binding sites are mainly located in the central nervous system,[19] although subtypes of receptor were recently identified in cardiomyocytes.[20] It is suggested that the mode of GHB action is driven by a central effect rather than a direct effect on peripheral cells (i.e., myocytes or keratinocytes). On one hand, the inhibitory effect of GHB on the dopaminergic system cannot directly explain the stimulation of the somatotropic axis. However, the indirect increase of serotonin levels after GHB administration could stimulate the secretion of GH through the effect on the dopaminergic system.[9] On the other hand, it is likely that GHB acts directly on the hypothalamus where specific binding sites have been found.[21,22] Based on more recent studies, it is suggested that the effects of GHB on GH secretion are mediated at least in part, by the effects of GHB on sleep.[14] It has been shown that major secretory pulse of GH occurs in timely association with slow wave sleep and particularly the first episode of a normal night.[23]

In the present study, modifications of UEA-1 or Mac 387 labeling in the epidermis were not observed with GHB treatment. Epidermis is then considered as not reactive: This supports the absence of keratinocyte damage due to GHB administration.

As it was reported that IGF1 levels were not altered when measured 9 hours after oral GHB ingestion,[14] we decided to administer GHB during several days and to set the evaluation time-point at D21 in order to have time enough to observe any change in keratinocyte profile.

Because our study is a pilot and preliminary study, GHB doses were empirically determined. No data related to GHB administration in burn patients are available in the current literature. In general anesthesia conditions, continuous infusion is about 15 to 30 mg/kg/h. However, in this study, a lower infusion rate was chosen, mainly to avoid hypernatremia, which is a common adverse event in case of prolonged administration. Patients of group C then received a GHB dosage similar to that described for sedation of critically ill patients[24] In group B, the lower limit of regular induction doses for general anesthesia (i.e., 50-100 mg/kg) was chosen: 50 mg/kg is a dose known to guarantee deep sedation associated with consistent electro-encephalographic changes.[25] Results of the present study may have been influenced by those arbitrary choices. Higher cumulative GHB dose may explain improvements observed in patients receiving continuous infusion in contrast to daily bolus administration.

Current guidelines for sedation management in critically ill patients recommend maintaining the lightest possible sedation levels.[26] As nonbenzodiazepine agents are suggested to be preferable, GHB may be an alternative option given its beneficial effects. For non-sedated patients, GHB could still be administered per os in the evening as in the treatment of narcolepsy. Further investigations should then consider higher dosage than in our group B as we did not demonstrate a sufficient effect in this group.

Finally, GHB does not seem to be the only drug that may stimulate keratinocyte proliferation. Similar pre-clinical data were recently published with valproate acid[27] or zoledronate.[28] Using well-known drugs away from their main indication could be in the next future an adjuvant treatment to promote wound healing. Depending on the drug, topical or systemic administration should be investigated in order, either to avoid adverse effects or to get benefit from its mode of action.

CONCLUSION

The present study is the first to consider GHB as a new option for promoting wound healing in human burn patients. This specific population experiences a hypermetabolic state with associated hyposomatotropism, leading to impaired wound healing. Even if GHB is not used anymore in daily practice in most of countries, it may be a safe adjuvant to a multimodal sedation strategy and it is available at low cost. Despite the limited number of included patients, the results of our preliminary trial are encouraging enough to warrant large prospective studies. Ideally, they should be multicentric and take advantages of regional and international collaborations in order to achieve a meaningful number of subjects. They should aim to confirm effectiveness of GHB to improve epithelialization,

to determine the optimal GHB dosage regimen and finally, to confirm the role of GHB in a global burn care strategy.

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