HYDROGEN GAS ENHANCES THE IN VITRO GROWTH OF HELICOBACTER PYLORI

HİDROJEN GAZİ HELİCOBACTER PYLORİ'NİN IN VİTRO ÜREMESİNİ ARTIRIR

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ABSTRACT

Purpose: *It is generally accepted that Helicobacter pylori (H. pylori) is microaerophilic, although the optimal atmospheric conditions for the growth of this organism have not been clearly defined. A few reports exist that point out the atmospheric requirements of H. pylori, namely reduced oxygen (O_2) tension and carbon dioxide (CO_2) enrichment. These studies and other personal observations provide evidence that the commercial hydrogen-producing kit systems are superior than oxygen-reducing ascorbic acid-based systems in supporting the growth of H. pylori. However, controversy persists whether the poor growth in ascorbic acid-based systems is attributable to the absence of hydrogen gas (H_2) or the inhibitory effect of ascorbic acid itself. This study was designed to elucidate the role of H_2 in growth of H. pylori.* ***Methods:*** *An airtight chamber was equipped with CO_2 - input and gas output valves to displace the air inside. KE-25 analytical O_2 sensor (Figaro Engineering Inc, Japan) was fitted inside the chamber to monitor the O_2 concentrations. An electrolysis cell was installed in the chamber system to generate H_2. Twenty H. pylori strains were cultured on brain-heart infusion agar supplemented with 7% horse blood and incubated simultaneously in a traditional microaerophilic jar and in the growth chamber described. A non-hydrogen-producing CampyGen CN35 kit was used to constitute a microaerophilic atmosphere in both systems. H_2 was produced in the growth chamber by the electrolysis of water. Following 72 hours of incubation, the growth characteristics of the cultures and the microscopic appearance of the cells were compared. Afterwards, the growth chamber was connected to a CO_2 tube to build up microaerophilic atmosphere by gas displacement. H_2 was produced by the electrolysis of water. Twenty H. pylori strains were cultured and incubated for 72 hours. At the end of this period the*

ÖZET

Amaç: *Mikroaerofilik olduğu kabul edilmesine karşın Helicobacter pylori (H.pylori) için optimal üreme koşulları tanımlanmamıştır. Organizmanın atmosferik gereksinimleri arasında azaltılmış oksijen (O_2) konsantrasyonu ve karbondioksitle zenginleştirmenin önemini vurgulayan az sayıda çalışma bulunmaktadır. Bu çalışmalarda elde edilen sonuçlar ve kişisel deneyimler, H. pylori'nin üretilmesinde hidrojen açığa çıkaran ticari kitlerin, oksijeni redükleyen askorbik asit içerenlere göre daha üstün olduğuna işaret etmektedir. Ancak, askorbik asitli kitlerle elde edilen zayıf üremenin bu sistemlerde hidrojen bulunmamasına mı, ya da askorbik asitin H. pylori üzerindeki inhibe edici etkisine mi bağlı olduğu açıklığa kavuşmamıştır. Hidrojen gazının H. pylori'nin üretilmesindeki rolünü incelemek amacıyla bu çalışma tasarlanmıştır.* ***Gereç ve Yöntem:*** *Hava geçirmeyen kapaklı bir kavanoza içerideki havanın CO_2 ile değiştirilmesini sağlamak amacıyla CO_2 girişi ve gaz çıkışı vanaları takılmıştır. Kavanozun içine oksijen konsantrasyonunun izlenmesi amacıyla KE - 25 analitik O_2 sensörü (Figaro Engineering Inc, Japan) ve H_2 üretilmesi için bir elektroliz pili yerleştirilmiştir. Yirmi H. pylori suşu %7 at kanlı beyin kalp infüzyon agarına ekilerek aynı anda hem tarif edilen kavanoz sisteminde, hem de geleneksel mikroaerofilik kavanozda inkübe edilmiştir. Her iki sistemde H_2 üretmeyen CampyGen CN35 kiti kullanılarak mikroaerofilik ortam sağlanmıştır. Tarif edilen kavanoz sisteminde su elektroliz edilerek H_2 elde edilmiştir. Yetmiş iki saatlik inkübasyonun sonunda her iki sistemde elde edilen üremeler koloni özellikleri ve hücre morfolojilerinin mikroskopik olarak incelenmesiyle karşılaştırılmıştır. Daha sonra tarif edilen kavanoz sistemi CO_2 tüpüne bağlanmıştır. Mikroaerofilik ortam kavanoz içindeki havanın CO_2 ile değiştirilmesiyle elde edilmiştir. Su elektroliz edilerek H_2 üretilmiştir. Yirmi H. pylori suşu beyin kalp infüzyon agar*

growth characteristics of the cultures and the microscopical morphology of the cells were assessed. ***Results:*** *Either CampyGen or gas displacement sufficiently reduced the O_2 tension in the growth chamber. Confluent growth and bacterial cells in predominantly spiral morphology were obtained in the growth chamber supplied with H_2 in both modes of action. In contrast, the H_2-free atmosphere of traditional microaerophilic jar yielded poorly grown cultures composed largely of coccoidal cells at the end of 72 hours.* ***Conclusion:*** *H_2 enhances the growth of H. pylori along with decreased O_2 tension.*

Key Words: *Helicobacter Pylori, Hydrogen Gas, Hydrogenase.*

plaklarına inoküle edilerek 72 saat inkübe edilmiştir. Bu sürenin sonunda koloni özellikleri ve bakteri hücrelerinin mikroskopik morfolojileri değerlendirilmiştir. ***Bulgular:*** *CampyGen ya da gaz değişimi yöntemlerinin her ikisi de kavanoz içindeki oksijen konsantrasyonunu yeterli derecede düşürebilmiştir. Hidrojen gazı eklenmiş inkübasyon ortamında her iki yöntemle de plağı kaplayıcı tarzda üreme ve ağırlıklı olarak spiral morfolojide hücreler elde edilmiştir. Hidrojen gazı bulunmayan geleneksel mikroaerofilik kavanoz sisteminde ise 72. saatin sonunda çoğunluğu kokkoidal morfolojideki hücrelerden oluşmuş zayıf üreme gösteren koloniler gözlenmiştir.* ***Sonuç:*** *Hidrojen gazı, azaltılmış O_2 konsantrasyonlarıyla birlikte H. pylori'nin üremesini artırıcı role sahiptir.*

Anahtar Kelimeler: *Helicobacter Pylori, Hidrojen Gazı, Hidrogenaz.*

INTRODUCTION

Helicobacter pylori is now defined as an important human pathogen with well established relations to active gastritis and peptic ulcer disease (1). It is generally accepted that this organism is microaerophilic, although the optimal atmospheric requirements have not been clearly defined. A few reports exist that point out the growth enhancing effect of reduced oxygen tension (5-10%) and increased carbon dioxide levels in vitro (2-4). Several methods have been widely used to grow *H. pylori* in the light of experience gained from the cultivation of related organisms, such as Campylobacter spp. (5). These include the use of hydrogen-producing kits, oxygen-reducing kits and incubation in a CO_2-enriched environment without O_2 reduction (2). These reports and personal observations demonstrate that commercial hydrogen-producing kits are superior to oxygen-reducing ascorbic acid-based systems in supporting the growth of *H. pylori* (2, 3, 5). Hydrogen gas is generally considered as a threat to laboratory safety, on account of its flammable and explosive properties (6). For this reason, "H_2 free", safe microaerophilic atmosphere generators are preferred over H_2-producing systems. However, controversy persists as to whether the poor growth afforded by these ascorbic acid-based systems is attributable to the lack of H_2 or the inhibitory effect of ascorbic acid itself (2, 7). In this study a controlled atmosphere growth chamber equipped with an oxygen sensor and an electrolysis cell was designed and built to establish the role of H_2 on the growth of *H. pylori*, in vitro.

MATERIALS AND METHODS

A 2.5 l steel jar with an airtight plastic lid was used to build the growth chamber. Carbon dioxide input and gas output valves were fitted on the lid of the jar. A KE-25 analytical oxygen sensor (Figaro Engineering Inc, Japan) was placed within. The calibration of the sensor was performed according to the manufacturer's instructions. An electrolysis cell was installed with the cathode side exposed to the inside, whereas the anode side connected to the outside of the jar. Water was electrolyzed to H_2 and O_2 at 25V in the presence of 0,5M aqueous sodium sulfate (Na_2SO_2). In this setting, H_2 was trapped inside the growth chamber, while O_2 was forced out of the jar.

Twenty *H. pylori* strains including the standard strain NCTC 11637 were included in the study. Two sets of experiments were carried out to assess the role of H_2 in the growth of *H. pylori*. A suspension of each freshly grown bacterial strain was prepared in sterile saline to a turbidity measurement equivalent to the 2 McFarland standard. The bacterial suspensions were plated on brain-heart infusion agar supplemented with 7% horse blood. Each strain was incubated simultaneously in a traditional microaerophilic jar system along with the growth chamber described in this study. Ten culture plates were incubated in both jars at a time. In both the jar and growth chamber, CampyGen CN35 (Oxoid, UK) ascorbic acid-based, "hydrogen gas free" kit was used in order to constitute a microaerophilic atmosphere. Paper towels with 10 ml of tap water were included in both systems to increase humidity. In the growth chamber, a system electrolysis cell was run for 36 minutes to obtain

250 ml of H_2. After 72 hours of incubation, the growth characteristics of the cultures and the microscopical appearance of *H. pylori* cells were assessed. The oxygen concentration of the growth chamber was measured at the end of the first hour allowing the gas mixture to stabilize, after 24 hours and 72 hours of incubation.

In the second set of experiments, the growth chamber was connected to a CO_2 tube to build up a microaerophilic atmosphere. In this setting, the air inside the chamber was displaced with CO_2 until the desired level of O_2 (6-7%) was reached and 250 ml (10% vol/vol) of H_2 was produced by electrolysis. Saline suspensions of 20 *H. pylori* strains were prepared and plated as described. After 72 hours of incubation, the growth characteristics of the cultures and the microscopical morphology of *H. pylori* cells were evaluated. Measurement of the O_2 concentrations were carried out at the end of hours 1, 24 and 72.

RESULTS

It was observed that each CampyGen CN35 sachet consistently reduced O_2 tension to 6-7% within 30 minutes in a 2.5 l jar as monitored by the oxygen sensor. In the first set of experiments, in which this kit was used to constitute a microaerophilic atmosphere, no growth failures were detected in both the growth chamber with H_2 and the traditional microaerophilic jar system. Confluent growth was observed in all of the cultures grown in the 10% H_2-supplied atmosphere, while a Gram stain of the colonies revealed predominantly spiral *H. pylori* cells. However, incubation under a microaerophilic atmosphere with no H_2 yielded poor growth, characterised by pinpoint or small colonies composed mainly of coccoidal cells as microscopical examination revealed. No decrease in O_2 concentrations in the growth chamber system were noted at the end of hour 1 and 24. However, the end-point measurements for O_2 concentration at the end of the incubation ranged between 0 and 0.4%.

Air-CO_2 displacement by using a CO_2 tube effectively reduced the oxygen tension to 6%, followed by the electrolysis of water to build up the desired H_2 concentration within the chamber. The incubation of *H. pylori* cultures under these atmospheric conditions afforded growth characteristics comparable to the microaerophilic conditions produced by the CampyGen kit supplied with H_2. At the end of the incubation period, confluent growth of the colonies was observed in all 20 cultures. Gram staining of the colonies demonstrated *H. pylori* cells mainly of a spiral morphology. There was no decrease in O_2 concentrations at the end of hours 1 and 24. The readings performed at the end of the incubation gave values ranging between 0 and 0.6%.

DISCUSSION

Aerobic and anaerobic respiratory pathways have been identified in *H. pylori* and it has been suggested that the organism requires oxygen in order to thrive. However, the presence of oxygen-sensitive enzymes such as pyruvate:acceptor oxidoreductase (POR) and a-ketoglutarate:acceptor oxidoreductase (OOR) restricts *H. pylori* to niches with reduced oxygen tension (4, 8). Isolation and cultivation procedures involving the use of CO_2 incubators, that merely offer a CO_2-enriched environment without O_2 reduction, usually yield unsatisfactory results (2). Reduced O_2 tension has been considered as a prerequisite for the successful cultivation of *H. pylori* (2, 9). In this study, the CampyGen CN35 kit provided a sufficient decrease in O_2 tensions in both H_2-supplied and H_2-free conditions, with no growth failures noted in any of the 20 *H. pylori* strains tested.

Henriksen et al. (2) reported that H_2-producing kits were significantly better than O_2-binding ascorbic acid-based systems in supporting the growth of *H. pylori*. However, it remained questionable whether the poor growth afforded by ascorbic acid-based systems is due to the lack of hydrogen or the inhibitory effect of ascorbic acid on *H. pylori*. Concurrent with this finding, in this study H_2 supplementation greatly enhanced the growth and viability of *H. pylori* cultures at the end of the incubation period when compared to incubation with CampyGen only.

Donelli et al. (4) reported that the viability of *H. pylori* cultures was dependent on the O_2 concentration in the environment. Oxygen deprivation rapidly decreases the spiral forms of

the bacteria. It was found that in anaerobic conditions, complete coccoidal conversion of the culture takes place over 7 days of incubation (4). In this study, it is suggested that the O_2 levels measured at the beginning and end of the incubation period reflect the O_2 consumption by a growing cell mass. Eventually, at the end of 72 hours, the O_2 inside the growth chamber becomes almost totally deprived. The lack a substrate for energy metabolism may be the most important factor responsible for the cessation of growth and conversion to coccoidal morphology in *H. pylori.*

Recently, *H. pylori* was found to contain membrane-bound H_2 uptake hydrogenase activity that is subject to anaerobic activation (9, 10). The organism is known to be able to use molecular hydrogen as a respiratory substrate in vitro. A hydrogenase mutant was less efficient in colonizing mice gastric mucosa. It has been suggested that H_2 produced by colonic bacterial activity may serve as an energy source for pathogenic bacteria (11). In this study, H_2 was shown to afford a protective effect on *H. pylori* cultures grown for 72 hours, as demonstrated by the growth characteristics and spiral morphology of the cells. Furthermore, the cultures grown without H_2 tended to fail to grow in consequent subcultures as opposed to those grown with H_2 supplementation (data not shown). Thus, the uptake and oxidation of H_2 may provide an alternative energy source for *H. pylori* below certain O_2 concentrations.

In conclusion, H_2 enhances the growth of *H. pylori* in vitro. The regulatory mechanisms that control H_2 metabolism remain to be elucidated.

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