POROUS CARBON PARTICLES FOR USE IN THE TREATMENT OR PREVENTION OF LIVER DISEASE

Field of the invention

The invention relates to the treatment or prevention of liver disease using porous carbon particles comprising micropores of diameter 2 nm or less and meso pores/small macropores of diameter 30 nm to 500 nm, but substantially no mesopores of diameter greater than 2 nm and less than 30nm, and substantially no large macropores of diameter greater than 500 nm. Also disclosed herein is the treatment or prevention of liver disease using porous carbon particles wherein at least 20% of the total pore volume is made up of pores having a mean diameter of from 2 to 200 nm. The invention also relates to methods for the treatment or prevention of liver disease, using such porous carbon particles.

Background to the invention

In the United States alone it is estimated that 60,000 people die each year of liver failure, whereas the donor pool remains constant at approximately 4000 with 16-18,000 on the waiting list. The odds of receiving a donor liver for subjects waiting on the list are only 1 in 8, yet there is no effective treatment or prevention available to extend the lifetime of this group of patients.

Liver failure results in multiple organ dysfunction and mortality rates are in the order of 80%. Bacterial-derived toxin and toxic metabolites such as acetaldehyde play key roles in disease pathogenesis. For example, gut-derived endotoxaemia and bacterial translocation play a central role in the pathogenesis of cirrhosis and its complications. However therapeutic options to target these factors are currently limited to long-term antibiotics with the attendant problem of infection with resistant organisms.

Orally administered adsorbent porous carbon particless have been used for centuries for the treatment or prevention of various disorders without any major side effects. Activated carbons are widely used to treat poisoning. A microporous carbon, AST-120 (available under the trade name KREMEZIN® from Kureha Corp., Japan) is used to treat patients with renal failure. However clinical trials evaluating the efficacy of AST-120 in the management of hepatic encephalopathy have proven negative.

Summary of the invention

The invention relates to the treatment or prevention of liver disease.

Figure 5: A. Acetaldehyde removal over time (500 μM (22 mg/L) Ac spike, n=3, mean +/-SEM, room temp, DIH derivatizing agent, HPLC ELS detection method); B. % Acetaldehyde (AT) adsorption by test carbon over time (7.2 mM spike, 0.1g/ml) (n=3, mean +/-SEM).

Figure 6: The effect of TE8 test carbon on bacterial growth measured using the BacTitre-Glo microbial cell viability assay to assess luminescence signal following direct contact with E coli over time (inoculum was $3.9x10^9$ bacteria ml^{-1}) (mean +/- sem, n=3).

Figure 7: The effect of TE7 test carbon on bacterial growth was measured using the BacTiter-Glo microbial cell viability assay to assess luminescence signal following direct contact with S. aureus over time (inoculum was $7.5 x10^7$ bacteria ml^{-1}) (mean +/- sem, n=3). The data suggests that the carbon does not kill these bacteria ex-vivo.

Figure 8: The effect of TE7 test carbon leachate on the growth characteristics of E coli over time was measured by increasing turbidity (540nm) using a Biscreen turbidometric analyser (mean, n=4). The data suggests that the carbon does not kill these bacteria ex-vivo.

Figure 9: The effect of TE7 test carbon leachate on the growth characteristics of B subtilis over time was measured by increasing turbidity (540nm) using a Biscreen turbidometric analyser (mean, n=4). The data suggests that the carbon does not kill these bacteria ex-vivo.

Figure 10: A. The removal of endotoxin by test carbons over time compared to no carbon controls (n=3, mean +/- SEM); B. The removal of endotoxin by test carbons over time compared to no carbon controls; higher endotoxin spike (0.1g, 200 EU/ml spike in SIF) (n=3).

Figure 11: Removal capacity of TE8 test carbon for TNF from SIF, expressed as an adsorption isotherm showing the amount of ~~carbon~~ TNF adsorbed per gram of carbon (μg) against the equilibrium concentration (mean, n=4).

Figure 12: Liver biochemistry – ALT: A significant reduction in alanine transaminase (ALT) was observed in the carbon treated BDL and BDL+LPS groups compared to untreated groups. Carbon treatment was associated with a reduction in ALT from 99 U/ml to 62 U/ml in BDL+LPS rats (p=0.0152) and from 71U/ml to 52U/ml (p=0.0422) in BDL rats. (Sham: Sham operated, BDL: Bile-duct ligation [studied 4 weeks after], LPS: animals administered lipolysaccharide intraperitoneally; BDL Carbon refers to the carbon treated group).

the BDL animals. (Sham: Sham operated, BDL: Bile-duct ligation [studied 4 weeks after], BDL Carbon refers to the carbon treated group).

Figure 20: Kupffer Cell LPS ROS Activity. Treatment with Carbon resulted in a reduction in endotoxin induced Kupffer cell generation of reactive oxygen species in the BDL animals treated with Carbon compared with the untreated group. The data provide a potential mechanism of reduced liver injury seen in the BDL animals. (Sham: Sham operated, BDL: Bile-duct ligation [studied 4 weeks after], LPS: animals administered lipolysaccharide intraperitoneally; BDL Carbon refers to the carbon treated group).

Figure 21: Intestinal Permeability. Gut permeability was normalised in the ~~in the~~ BDL animals treated with Carbon compared with the untreated group. The data demonstrates that the carbon treated group normalized the increased permeability that was observed in the BDL animals. (Sham: Sham operated, BDL: Bile-duct ligation [studied 4 weeks after], BDL Carbon refers to the carbon treated group).

Figure 22: Markers of Bacterial Translocation (Portal venous endotoxin). Treatment with Carbon markedly reduced endotoxemia, altered gut permeability and bacterial translocation.

Figure 23: Distant Organ Effects (Hepatic Encephalopathy). Treatment with carbon resulted in reduction in brain water and serum ammonia levels.

Figure 24: Markers of Bacterial Translocation (Bacterial PCR Positivity). Treatment with Carbon resulted in a marked reduction in bacterial translocation.

Figure 25: Portal Venous Cytokines. A non-significant reduction in portal venous IL-4 and IL-10 was observed with carbon therapy.

Figure 26: Histology of Colon. Histology of the ileum, jejunum and colon remained unaffected following treatment with the Carbon showing that the treatment was safe to the intestinal mucosa. (Sham: Sham operated, BDL: Bile-duct ligation [studied 4 weeks after], BDL Carbon refers to the carbon treated group).

Figure 27: Serum creatinine. The rise in serum creatinine representing renal function deteriorated in the BDL animals following administration of LPS, which was prevented in the BDL animals treated with carbon suggesting that it reduces acute kidney injury of cirrhosis. (Sham: Sham operated, BDL: Bile-duct ligation [studied 4 weeks after], LPS: animals administered lipolysaccharide intraperitoneally; BDL Carbon refers to the carbon treated group).

Figure 28: Brain water (%). In the BDL animals treated with LPS brain water increased which was prevented in the animals treated with the carbons suggesting that treatment of BDL animals reduces the brain complication of cirrhosis and therefore hepatic encephalopathy. (Sham: Sham operated, BDL: Bile-duct ligation [studied 4 weeks after], LPS: animals administered lipolysaccharide intraperitoneally; BDL Carbon refers to the carbon treated group).

Figure 29: Bacteroides/total bacteria ratio in the stool. The data suggests that the bacterioides species is increased in the stool of BDL animals which is reduced towards normality in the carbon treated BDL animals but the total number of bacteria remain the same. (Sham: Sham operated, BDL: Bile-duct ligation [studied 4 weeks after], BDL Carbon refers to the carbon treated group).

Figure 30: Firmicutes/total bacteria ratio in the stool. The data suggests that the fermicutes species is reduced in the stool of BDL animals which is increased towards normality in the carbon treated BDL animals but the total number of bacteria remain the same. (Sham: Sham operated, BDL: Bile-duct ligation [studied 4 weeks after], BDL Carbon refers to the carbon treated group).

Figure 31: Firmicutes/Bacteriodetes ratio in the stool. The data suggests that the fermicutes to bacteroides species ratio is reduced in the stool of BDL animals which is increased towards normality in the carbon treated BDL animals. (Sham: Sham operated, BDL: Bile-duct ligation [studied 4 weeks after], BDL Carbon refers to the carbon treated group).

Figure 32: Weight. Obese mice lose weight on treatment with the carbons towards normality in Leptin deficient Ob/Ob mice. The data suggest that the carbons may be a useful treatment of obesity. (Ob-: Heterozygote for the ob gene; Ob-/Ob-: Homozygote for the Ob/Ob gene; +C: groups treated with Carbon for 4 weeks).

Figure 33: ALT. Treatment of obese (Ob/Ob) mice with carbon results in a reduction in liver injury suggesting that carbon may be a treatment for non-alcoholic fatty liver disease. (Ob-: Heterozygote for the ob gene; Ob-/Ob-: Homozygote for the Ob/Ob gene; +C: groups treated with Carbon for 4 weeks).

Figure 34: Liver Histology. Treatment of obese mice with carbon results in a reduction in hepatic fat accumulation and inflammatory cell infiltration. (Ob-: Heterozygote for the ob gene; Ob-/Ob-: Homozygote for the Ob/Ob gene; +C: groups treated with Carbon for 4 weeks).

Figure 35: Kupffer cell population (F4/80+/CD68-/CD11b+):. Treatment of obese mice with carbon results in a reduction in the activation of the Kupffer cells. (Ob-: Heterozygote for the ob gene; Ob-/Ob-: Homozygote for the Ob/Ob gene; +C: groups treated with Carbon for 4 weeks).

Figure 36: Kupffer cell population (F4/80+). Treatment of obese mice with carbon results in a reduction in the activation of the Kupffer cells. This provides a possible mechanism by which liver injury is reduced in the Ob/Ob mice treated with the carbon. (Ob-: Heterozygote for the ob gene; Ob-/Ob-: Homozygote for the Ob/Ob gene; +C: groups treated with Carbon for 4 weeks).

Figure 37: Kupffer cell population F4/80+ LPS: ROS production. Treatment of obese mice with carbon results in a reduction in the production of reactive oxygen species by the Kupffer cells. (Ob-: Heterozygote for the ob gene; Ob-/Ob-: Homozygote for the Ob/Ob gene; +C: groups treated with Carbon for 4 weeks).

Figure 38: Liver Histology (Collagen staining). In the half methionine choline deficient diet (HMCD) model of non-alcoholic fatty liver disease, Carbon reduces histological fibrosis suggesting that Carbon treatment may be a treatment for hepatic fibrosis. (HMCD untreated: Diet alone; HMCD Carbon: Diet + Carbon treatment for 4 weeks).

Figure 39: Liver; Gene Expression for markers of fibrosis. In the half methionine choline deficient diet (HMCD) model of non-alcoholic fatty liver disease, Carbon reduces the gene expression of Collagen A2 and TGFB1. The data support that Carbon may be a treatment for the prevention of hepatic fibrosis (HMCD~~Θ~~ untreated: Diet alone; HMCD~~Θ~~ C4: Diet + ~~C4~~ carbon treatment for 4 weeks).

Figure 40: ALT. In the half methionine choline deficient diet (HMCD) model of non-alcoholic fatty liver disease, administration of Carbon reduces liver injury suggesting that carbon may be a treatment for non-alcoholic fatty liver disease. (HMCD: Diet alone; HMCD +C: Diet + Carbon treatment for 4 weeks; WT: Untreated control).

Figure 41: Liver Histology (H&E staining). In the half methionine choline deficient diet (HMCD) model of non-alcoholic fatty liver disease, Carbon reduces fat accumulation in the liver suggesting that carbon may be a treatment for non-alcoholic fatty liver disease. (HMCD untreated: Diet alone; HMCD Carbon: Diet + Carbon treatment for 4 weeks).

Figure 42: Liver Histology (Steatosis scores). In the HMCD model of non-alcoholic fatty liver disease, Carbon reduces fat accumulation suggesting that carbon may be a treatment for non-alcoholic fatty liver disease. (HMCD untreated: Diet alone; HMCD Carbon: Diet + Carbon treatment for 4 weeks).

The present inventors have investigated the capacity of porous carbon particles having controlled porosity to adsorb biological molecules and their application in treating or preventing liver disease, thus providing an alternative strategy to treatment using conventional antibiotics.

In one aspect, therefore, the invention relates to porous carbon particles ~~particles~~ for use in the treatment or prevention of liver disease, wherein the particles comprise mesopores of diameter 2-50 nm and small macropores of diameter 50 nm and above. In another embodiment, the porous carbon particles of the present invention comprise micropores of diameter 2 nm or less and mesopores/small macropores of diameter 30 nm to 500 nm, but substantially no mesopores of diameter greater than 2 nm and less than 30nm, and substantially no large macropores of diameter greater than 500 nm.

The present inventors have found that such non absorbable porous carbon particles having a controlled porosity to provide a relatively high proportion of pores in the meso-to-macroporous range, or in the micro- and small macro ranges, are suitable adsorbants of the pathogenic mediators discussed above and are able to modulate the function of Hepatic Kupffer cells which produce damaging free radicals, possibly through reduction in the translocation of toll-like receptor ligands. The porous carbon particless are non-absorbable and therefore mediate their effect locally at the gut-barrier interface. However unlike conventional non-absorbable antibiotics, the porous carbon particles of the present invention have been shown not to affect detrimentally the growth of bacteria which are important to maintain gut ecology.

Conventionally produced activated carbon (e.g. granular activated carbon) is normally microporous, having pores of diameter less than 2 nm (IUPAC definition), with little or no pore volume in the mesopore (2-50 nm) or macropore (greater than 50 nm) range.

The porous carbon particles for use in the present invention may have 20% to 90% of the total pore volume is made up of pores having a mean diameter of 2 nm or less (micropores), and 75% or more of the remainder of the total pore volume (i.e. the pore volume made up of pores having a mean diameter greater than 2 nm) is made up of pores having a mean diameter of from 30 nm to 500 nm (mesopores/small macropores).

Thus, the porous carbon particles may have a bimodal distribution of pore sizes whereby the total pore volume is distributed between the micropore and large mesopore/small macropore ranges, with substantially no mesopores of diameter less than 30 nm or large macropores. The presence of large macropores is preferably

resulting from the process of US2010/0086469, which comprises supplying the material to an externally fired rotary kiln maintained at carbonizing and activating temperatures, the kiln having a downward slope to progress the material as it rotates, the kiln having an atmosphere free of oxygen provided by a counter-current of carbon dioxide or steam, and annular weirs being provided at intervals along the kiln to control progress of the material.

Alternatively the resin beads can be carbonised and activated on a smaller scale using a batch furnace. Here the carbonisation and activation may be carried out as separate steps where the carbonisation takes place in carbon dioxide at ~800C and the activation in carbon dioxide at between 850 and 950C or in steam at between 700 and 850C.

For the purposes of this invention it is preferred to use carbon dioxide as the activating medium although it is also possible to use other media.

Use of Porous Carbon Particles in the Treatment of Liver Disease

The porous carbon particles described above are useful in the treatment or prevention of liver disease. Liver failure is the final stage of liver disease. Liver failure is divided into types depending on the rapidity of onset. Acute liver failure develops rapidly, but chronic liver failure may take months or years to develop. By definition, liver failure occurs when the liver is so diseased, and functioning so poorly, that encephalopathy is evident. Any progressive liver disease can result in liver failure; examples include: acetaminotophen toxicity, cirrhosis, viral hepatitis, and metastatic cancer of the liver. Other signs of liver disease such as jaundice, ascites, fetor hepaticus, and failure of coagulation indicate that the liver is having trouble performing its normal physiological duties, but it is not termed liver failure until the mental status changes appear.

The prognosis for patients with liver disease is difficult to estimate because the condition has many causes.

Accordingly, the present invention may relate to treatment or prevention of an individual whose liver is decompensated or which shows hepatic encephalopathy. The individual's liver may be in the compensated state. The individual may have chronic liver disease. The individual may have liver cirrhosis, for example with or without alcoholic hepatitis. The individual may have acute liver failure. The individual may have hepatic encephalopathy.

around 140°C followed by substantial evolution of gas. The further heating to 150-155°C for 15-20 minutes ensured the completion of curing. The mixture was cooled and the resulting beads were separated from the oil by filtration or centrifugation Ethylene glycol was removed from the resin either by multiple hot water extraction or by drying in vacuum (120°C at 50 mm Hg). In the above procedure, compared to Example 3 of WO 02/12380, the hexamine content has been increased to 15-20pbw per 100pbw of novolac from the previously exemplified 9 pbw, and the temperature of the oil into which the resin solution is poured is increased from 115-120°C to 150-155°C, and "flash" cure is brought about rather than a "slow" cure as previously exemplified.

Water-washed wet, dried or vacuum-dried resin beads were heat treated to produce carbon materials. A typical procedure comprised but is not restricted to carbonisation in a flow of carbon dioxide with temperature ramping from ambient to 800°C at 3°C/min, classification by particle size and further "physical" activation of selected fraction in carbon dioxide flow at 900°C. Many variations of this routine known in the art may also be applied. The degree of activation in these samples was approximately 30%.

The meso/macro pore size distribution in the resulting carbons is pre-determined by the porosity of the resin-precursor, which is controlled by the content of the solvent/pore former and the degree of activation of the resulting carbon. **Table 1** below gives details of four resin compositions that are precursors to the meso/macro porous carbons, as illustrated by nitrogen porosimetry and mercury porosimetry tests of the activated materials. The predominantly micro - macroporous materials, TE7 and TE8 were used in the subsequent biomedical testing and have a very similar macropore structure. The TE3 and TE5 materials, which have smaller pores in the meso/macro domain are for illustration and give inferior performance in the adsorption of the larger molecules such as TNFα.

The particle size distribution of resulting resin beads depends on various parameters including but not restricted to the type of stirring tool, stirring rate, viscosity of the resin solution, concentration of the dispersing agent, resin solution to oil ratio and temperature of the dispersion. Though the distribution is typically broad the size of the predominant fraction could effectively be shifted between ~10 micron and ~1 mm.

Table 1

Table 2 Pore structures for CO2 Activated TE8 Carbons

			Total Vol (0.99)	Incremental Pore Volume		
Material	Density	BET Area	0.99	0-2nm	2-50nm	>50nm
	g/cm3	m2/g	cm3/g	cm3/g	cm3/g	cm3/g
TE8 Unactivated		534	0.9	0.101	0.136	0.56
29% activated	0.22	1041	1.31	0.34	0.22	0.66
47% burn ~~off~~ off	0.17	1432	1.67	0.96	0.355	0.81

The change in the macropore volume of the carbon with activation is an artefact of the nitrogen adsorption method but should be >0.5cm3/g. The larger pore structures should be measured using mercury porosimetry. These are shown for TE7 and TE8 activated to 40% burn off in figure 2. The larger pores at >38000nm are due to interparticle voids between the beads, not to any internal porosity within the beads. The TE7 and TE8 particles have pore diameter peaks at 88 nm and 91 nm respectively, surface area of 1499 m^2/g, pore volume of 1.36 cm^3/g, bulk density of 0.2 g/cm^3, particle size of 240-500 μm and 40% degree of activation. The absence of pores both above the main peak (>300nm) is readily apparent as is the essential absence of the pores in the mesopore (2-50nm) domain for both materials. Mercury cannot provide data in pores below ~6nm due to the pressure involved.

Unless otherwise specified the porous carbon particles used in the following *in vitro* and *in vivo* studies were the activated TE7 particles described above.

In vitro studies

Investigating the effect of direct contact incubation of test carbons on bacterial metabolism

Carbon materials (TE7 or TE8 as specified) were weighed into glass universal bottles and dry heat sterilised for 2 hours at 80°C. 1ml of phosphate buffered saline (PBS) was added to each 0.1g of material and samples were incubated at 37°C for 1 hour while shaking at 120rpm. Tryptone soya broth (TSB) was inoculated with *Escherichia coli* (NCTC 10418) or *Staphylococcus aureus* (NCTC 6571) and incubated overnight at 37°C while shaking at 120rpm. The bacterial suspension was pelleted by centrifugation and re-suspended in 1 ml PBS. The absorbance of the suspension was measured at 540nm and the suspension concentration was adjusted to give a value of 0.5. This dilution factor was then used to prepare a bacterial suspension in TSB. A

viability count was carried out on the *E coli* suspension by serial dilution and plating onto agar plates. The inoculum (1ml) was added to each material and the samples were incubated at 37°C while shaking at 120rpm. At timed intervals of 30mins, 2 and 6 hours 100µl of bacterial suspension was removed from each sample and placed into the wells of a 96 well plate. Samples were lysed and analysed for ATP content as a measure of cell metabolism using the BacTiter-Glo microbial cell viability assay (Promega).

Investigating the effect of carbon leachate on bacterial metabolism

Carbon materials (TE8 and commercially available ACTIDOSE charcoal indicated for poisoning) were weighed into glass universal bottles and dry heat sterilised for 2 hours at 120°C. 2ml of phosphate buffered saline (PBS) was added to each 0.2g of material and samples were incubated at 37°C for 24 hrs while shaking at 120rpm. TSB was inoculated with *E coli* (NCTC 10418) or *Bacillus subtilis* and incubated overnight at 37°C while shaking at 120rpm. The bacterial suspension was pelleted by centrifugation and diluted in TSB to give a final approximate concentration of $1x10^9$ bacteria ml^{-1}. In a 100 well Bioscreen plate, 100µl of extract, 100µl of inoculum and 100µl of TSB were added and bacterial growth was measured in a Bioscreen turbidometric analyser monitoring bacterial number at 540nm for 72 hrs.

Investigating test carbon adsorption of endotoxin over time

Endotoxin removal by TE8 test carbon was measured using a limulus amebocyte lysate (LAL) endosafe endochrome-K test (Charles River Laboratories UK) and Tecan Sunrise incubating plate reader with endoscan-V software. Depyrogenated glassware and endotoxin free plastics were used to minimise endotoxin contamination. TE8 test carbon beads underwent dry heat sterilisation at 250°C for three hours. Carbons were pre-wetted with simulated intestinal fluid (SIF). SIF was made immediately prior to each experiment according to the United States Pharmacopoeia 26 recipe without pancreatin and using LAL reagent water. A standard lipopolysaccharide (LPS) solution derived from *E coli* 055:B5 was prepared at a concentration of 200 EU ml^{-1} in SIF. SIF was removed from the carbons by aspiration and endotoxin spiked SIF was added to each test carbon at a volume to weight ratio of 10 ml per gram of carbon. Test samples and positive controls without carbon were incubated at 37°C with shaking and 450 µl of sample was removed at time points of 0, 15, 30, 45 and 60 minutes. The endotoxin

concentration of each sample was calculated according to manufacturer's instructions against a standard curve prepared using dilutions from 0.005 to 50 EU ml^{-1}.

Investigating test carbon adsorption of TNF

Different weights of carbon bead (TE8 test carbon) ranging from 0.001 to 0.005g, were placed into sterile labelled eppendorf tubes in quadruplicate and were pre-wetted in 1 ml of SIF in a shaking incubator at 37°C for 2 hours. Eppendorfs were centrifuged at 8000rpm for 3 minutes. Supernatant was removed and SIF spiked with 10ng/ml of recombinant TNF was added. TE8 test carbon adsorbents were incubated at 37°C while shaking at 90 rpm for 24 hours. Samples were centrifuged at 8000rpm for 3 minutes and supernatant was collected and stored at -20°C. Samples were diluted in assay diluent before measurement of TNF concentration by ELISA according to the manufacturer's instructions (BD Biosciences).

Investigating the effect of carbon adsorption on acetaldehyde

Different weights of carbon bead (TE8 test carbon) ranging from 0.001 to 0.005g, were placed into sterile labelled eppendorf tubes in quadruplicate and were pre-wetted in 1 ml of SIF in a shaking incubator at 37°C for 2 hours. 7.2 mM spike, 0.1g/ml) of acetaldehyde was added (n=3, mean +/-SE). Eppendorfs were centrifuged at 8000rpm for 3 minutes. Acetaldehyde (AT) adsorption was measured using a derivatisation method with 2-diphenylacetyl-1,3-indandione-1-hydrazone (DIH) and detection by HPLC. (Figure 5B)

***In Vivo* studies**

All animal experiments were conducted according to Home Office guidelines under the UK Animals in Scientific Procedures Act 1986. Male Sprague-Dawley rats (body weight 280-300g) were used (Charles River Laboratories UK Ltd.). All rats were housed in the unit and given free access to standard powdered rodent chow and water, with a light/dark cycle of 12 hours, at a temperature of 19°-2C to 23°-2C and humidity of approximately 50%.

Bile duct Ligation Model

Under halothane anaesthesia 131 male Sprague-Dawley rats underwent bile duct-ligation or sham biliary surgery. Rats were pair-fed powdered chow +/- pre-hydrated Mastcarbon (250-500μm) (TE7) at a dose of 0.4g/100g body weight per day from two weeks after bile duct ligation until completion of the experiment at 4-5 weeks from initial surgery. Intraperitoneal *Klebsiella* lipopolysaccharide (LPS) (0.33mg/kg) was administered to 4 subgroups 3.5 hours prior to completion of study. The following groups were studied: Sham (n=15), Sham + carbon (n=17), Sham + LPS (n=11), Sham+LPS+carbon (n=10), BDL (n=22), BDL + carbon (n=25), BDL+LPS+ carbon (n=10), BDL+LPS+carbon (n=16).

Intestinal permeability assays

Intestinal permeability assays were conducted 1 day prior to completion of the experiment. Animals were placed in metabolic cages for overnight acclimatisation. Baseline urine samples were collected in cryotubes and stored at -70°C. 0.6ml of a solution of lactulose (277mM), L(+)-Rhamnose (10mM) and 3-methyl-o- pyranose (2.0mM) was then administered by gavage and urine collected for the subsequent 5 hours. Urine samples were analysed using mass spectroscopy. Animals were returned back to their group cages for re-acclimatisation and fasted prior to termination.

Haemodynamic measurements and sample collection

Under halothane anaesthesia (5ml/min induction 2ml/min maintenance) an internal carotid catheter (0.96 outer diameter Portex fine-bore polythene tubing, Scientific Laboratory Supplies Ltd., Nottingham, UK) was inserted as previously described. The catheter was held in place for the duration of the study by both proximal and distal holding sutures. The catheter was transduced and mean arterial pressure determined. A laparotomy was then performed under sterile conditions and a catheter placed in the portal vein. Arterial and portal venous catheters were transduced. Concomitant arterial and portal venous plasma was collected aseptically into lithium heparin and EDTA tubes until a state of exsangaguination was achieved. 5ml of ice-cold PBS was then perfused into the liver to achieve organ blanching. The liver was extracted and placed in 10ml of ice cold PBS. Plasma was centrifuged at 3,500rpm for 10 minutes at 4°C. The supernatant was transferred immediately to cryotubes and stored at -70°C.

Duodenum, mid-jejunum, terminal ileum and ascending colon was collected and stored in formaline and electron microscopy preservation solution (200mM sodium cacodylate, 4% gluteraldehyde, pH=7.2-4). Histological specimens were collected with a bed of mesentery with no attempt to irrigate or decontaminate the lumen. Samples were also collected from all four sites following decontamination of the lumen and irrigation with saline solution. These samples were transferred immediately to cryotubes and stored at -70°C. Liver, kidney and brain tissue was also collected and stored in formaline and cryotubes and stored at -70°C.

Isolation of Liver Non-Parenchymal Cells

Perfused liver tissue was dissected with a scapel and homogenized in Hanks balanced salt solution (with calcium and magnesium + collagenase 0.01% and DNAse I (0.01%). The homogenate was transferred to a 50ml Falcon tube and incubated at 37°C prior to filtration through a 100mcm cell strainer. This was then centrifuged at 500rpm for 5 minutes at 4°C and the supernatant subsequently centrifuged at 2000rpm for 10 minutes at 4°C. The supernatant was discarded and the pellet resuspended in PF4 (HBSS with no calcium or magnesium, DNAse I 0.01%, bovine serum albumin (0.25%)) and centrifuged at 2000rpm for 10 minutes at 4°C. The pellet was then resuspended in 3.9ml of RPMI 1640 and mixed gently with 2.1ml (RPMI and optiprep 22%). RPMI was then layered on top followed by 25 minute centrifugation 2800rpm without brake at 4°C. The non-parenchymal cells were isolated from the interface, resuspended in an equivalent volume of PF4 and centrifuged at 2000rpm at 4°C for 10 minutes. 10 x 10^6 cells were used in all subsequent assays.

Kupffer cell phagocytic function

The cells were centrifuged at 2000rpm for 5 minutes at 4°C and the supernatant discarded. 200ul of latex beads containing media were added to the pellet and incubated at 37°C in the dark for 20 minutes. 5ml of ice cold PBS was then added and centrifuged at 2000rpm for 5 minutes at 4°C. The pellet was then washed with 5ml of cold PBS and centrifuged. Fc blocker was then added and incubated for 10 minutes at 4°C. Anti-CD163 antibody was then added and incubated for 30minutes at 4°C in the dark.

Kupffer cell reactive oxygen species (ROS) production

20ug/ml of *E.coli* endotoxin was added to 1x 10^6 non-parenchymal cells sample and incubated for 30minutes at 37°C. ROS inducer at a final concentration of 200-500uM was used for the positive control. The samples were then centrifuged at 500g for 5 minutes and the supernatant discarded. The cells were then resuspended in 5ml of wash buffer, centrifuged at 500g for 5minutes and the supernatant removed. The cells were re-suspended in 500ul of ROS detection solution and incubated for 30 minutes at 37°C in the dark. Following centrifugation, the cells were resuspended in 100ul of FACS buffer, Fc blocker added (1:25) and incubated for 10minutes at 4°C. Anti-CD163 antibody was added and the cells incubated for 30minutes at 4°C in the dark. The cells were then washed with 1ml of FACS buffer, centrifuged and resuspended in 100mcl FACS buffer solution.

Cytokine analysis

Portal venous TNFα, IL-4, IL-10 levels were determined using the BD™ Cytometric Bead Array (CBA) kit. 50μL of the mixed capture beads were added to each assay well of a pre-wetted plate. 50 μL of standard or sample was then added to the assay wells. The plate was agitated for 5 minutes using a digital shaker at 500 RPM and the plate incubated for 1 hour at room temperature. 50 μL of mixed PE detection reagent was then added to each assay well. The plate was then agitated for 5 minutes using a digital shaker at 500 RPM and incubated at room temperature for 2 hours. The plate was vacuum aspirated until the wells were drained. 150μL of wash buffer was added to each assay well. The plate was then agitated on a digital shaker at 500 RPM for 5 minutes to resuspend the beads. The samples were then analysed by flow cytometry and data analysed using FACS Diva software.

Endotoxin measurement

The chromogenic limulus amoebocyte lysate kinetic assay (Charles River Laboratories) was used for the detection of endotoxin. Portal venous plasma (100mcl) was diluted 1:10 with endotoxin-free water and incubated at 75°C for 30 minutes. 100mcl of sample and 100mcl of LAL reagent were mixed in a 96-well plate and analysed at 405nm with spectrophotometer using the Endoscan V software. Results are expressed as EU/ml.

Neutrophil isolation

Whole blood (4ml) from healthy volunteers was layered over 5ml of Polymorphoprep and spun for 30 minutes at 400g at room temperature. Neutrophils were harvested from the second interface and washed with phosphate buffered saline. Neutrophils were counted and resuspended in PBS at a density of $5x10^5$ in 50mcl±50mcl of cell suspension and 50mcl plasma were used per assay. Viability was assessed.

Neutrophil function

Effect of co-incubation of portal venous plasma on oxidative burst and phagocytosis of normal human neutrophils was determined using Phagoburst and Phagotest assays. The Phagoburst kit (Orpegan Pharma) was used to determine the percentage of neutrophils which produce reactive oxidant species as previously described (FACS CantoII, BD bioscience). The Phagotest (Orpegan Pharma was used to measure phagocytosis by using FITC-labeled opsonised *E.coli* bacteria as described before. After incubation of cells and plasma for 90 minutes they were washed with PBS and incubated with CD16-PE (3mcl) (Immunotools). The mean fluorescent intensity of the respective antibodies on neutrophils were analysed by flow cytometry (FACS Canto II, BD bioscience).

Biochemical analysis

Biochemical profile was determined using standard techniques (COBAS).

Histological analysis

Liver tissue was processed in accordance with standard protocol and Haematoxylin and Eosin together with Sirius Red staining was performed. Histological staging was conducted by a consultant histopathologist using a 14 point secondary biliary cirrhosis scoring system. Sirius red staining was quantified using computer assisted digital image analysis. Collagen proportionate area was determined using Zeiss KS300 image analysis software. TLR-4 expression in the colon, liver and kidney was determined by immunohistochemistry.

Brain water analysis

Brain water was quantified in accordance with standard protocol. 100g of brain tissue was placed in 100°C incubator for 24 hours. The percentage water loss was calculated.

Statistical Analysis

Data are expressed as mean± standard error of the mean (SEM). Software used included Graphpad Prism 5.0 (GraphPad software, Inc., San Diego, CA).

Results

***In Vitro* studies**

Example 1: Investigating the effect of direct contact incubation of test carbons on bacterial metabolism

The direct incubation of TE8 test carbon with bacterial suspensions of either E. coli or S. aureus in TSB indicated that the TE8 carbon does not affect bacterial growth for either species following direct contact of 4 to 6 hours (Figures 6 and 7). The luminescence measurement is an indirect measure of cell viability and cell number via the determination of bacterial ATP. Levels of luminescence, reflective of bacterial metabolism on sample contact, were comparable to the no adsorbent control for both species over the time course of the experiment. In contrast, the control commercial ACTIDOSE oral carbon greatly reduced the luminescence signal after only 30 minutes incubation of bacteria with the carbon.

Example 2: Investigating the effect of carbon leachate on bacterial metabolism

The direct incubation of TE7 carbon leachate with bacterial suspensions of either E coli or Bacillus subtilis in TSB indicated that the TE7 carbon leachate does not affect bacterial growth for either species for up to 72 hrs incubation (Figures 8 and 9). The optical density (OD) measurement is an indirect measure of bacterial number. OD values for the TE7 sample were comparable to the no adsorbent control for both species over the time course of the experiment.

Example 3: Investigating test carbon adsorption of endotoxin over time

Distant organ effects

No significant difference in renal histology with routine staining (H&E and PAMS). TLR-4 expression in the kidney was not significantly different. Serum creatinine, as a reflection of renal function was lower in the BDL+LPS group treated with carbon but not significantly different (Figure 30). Brain water was lower in the carbon-treated BDL+LPS group but not statistically significant (Figure 30). No evidence of carbon embolisation was observed at either a macroscopic or microscopic level.

Discussion

We demonstrate *in vitro* that micro/mesoporous carbons have the optimal porosity to bind intraluminal factors relevant to the pathogenesis of chronic liver disease. A high affinity for free endotoxin was demonstrated but with no significant affect on bacterial growth kinetics. Micro-/mesoporous carbons are therefore acting as endotoxin adsorbeants without antibiotic effects. Translocation of bacterial products in the absence of viable bacteria is a recognized phenomenon in cirrhosis. Clinical studies have shown that culture-negative bacterial PCR positivity in serum and ascites is predictive of survival. Translocation of free intraluminal endotoxin has been demonstrated in BDL rats and shown to drive systemic endotoxaemia, implicated in the pathogenesis of ACLF. Therefore micro-/mesoporous carbons, with the capacity to bind free endotoxin, have the potential to diminish this process. Furthermore, in not influencing bacterial growth kinetics, carbons are not likely to be associated with the side effects attributed to antibiotic therapy. These include antibiotic-induced dysbiosis with a shift in the commensal flora towards resistant bacterial populations in the gut with potentially more deleterious effects. In addition, antibiotics result in endotoxin generation and have a variable influence on endotoxin kinetics dependent on antibiotic class. In the absence of antibiotic activity, this effect will not be observed with carbon therapy.

Endotoxaemia is known to drive a dysregulated inflammatory response in cirrhosis. The *in vitro* data in this study confirms that micro-mesoporous carbons have a high affinity for pro-inflammatory cytokines of potential relevance to the pathogenesis of cirrhosis and acute on chronic liver failure. Clinical studies describe an association of portal venous cytokine levels with the natural history of disease including portal

of evidence implicate endotoxin in pathogenesis. That increased oxidative burst was conferred by a humoral factor strongly implicates endotoxin in pathogenesis. A reduction in portal venous IL-4 and IL-10 was also observed. IL-4 has been implicated in Kupffer cell activation yet IL-10 has been implicated in dampening Kupffer cell responses within the context of the adiponectin/interleukin-10/heme oxygenase-1 pathway. It is perhaps not unsurprising if carbon is indiscriminant in cytokine binding that both pro- and anti-inflammatory cytokine responses are diminished in parallel. In fact patients manifesting with acute-on-chronic liver failure exhibit pronounced pro-and anti-inflammatory responses concurrently. There is a potential role for diminishing both of these within the portal circulation without impacting on systemic immune function. As frequently observed in biological systems, there is considerable heterogeneity in the values observed rendering the trends non-significant and therefore require further evaluation. (Figures 23 to 26.)

While carbon has no significant effect on *E. coli* and *S. aureus* growth kinetics in *in vitro* studies (Figures 7 and 8), a modulation in faecal bacterial populations was demonstrated *in vivo* following carbon therapy (Figures 31 to 33). A marked effect was observed with *bacteroides* populations. Carbon may therefore influence growth kinetics of non-E.coli bacteroides or influence the composition of the gut microflora by binding bacterial metabolites or other intercellular signaling molecules

A reduction in liver smooth muscle actin expression was observed in carbon-treated animals. This suggests that the downstream effects of carbon treatment include a modulation of stellate cell function. Collagen proportionate area as a measure of fibrosis was not found to be significantly different following carbon treatment possibly because the animals were only treated for the final two weeks but the reduction in smooth muscle actin suggests that carbon treatment may result in reduced hepatic fibrosis. Taken together, this data suggests that the effects of carbon on portal haemodynamics are mediated at a sinusoidal level. Given the observed effects on Kupffer cell population and function, we postulate that micro-/mesoporous carbon therapy results in diminished translocation of bacterial products and consequent inflammatory response resulting in diminished Kupffer cell priming, ROS production and stellate cell activation and therefore fibrosis.

A significant improvement in final body weight was observed in carbon-treated compared to untreated BDL rats. No significant difference in final body weight was observed between the sham groups. Weight loss in cirrhosis is attributed to an increased catabolic