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Research Article

Benefits and Biosafety of Use of Buckypaper for Surgical Applications: A Pilot Study in A Rabbit Clinical Trial Model

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Abstract

Background: One of the main problems related to prosthetic abdominal surgery is mesh fixation. Suture line tension, mesh separation, displacement, or improper application of stitches are the leading causes of complications, including seroma, postoperative pain, and recurrence. A surface able to adhere firmly to living tissue represents an effective alternative to conventional perforating fixations. As a bio-adhesive tape, we report experimental evidence on the potential applicability of the BuckyPaper (BP), a felt composed of entangled multi-walled carbon nanotubes.

Matherial and methods: BP is implanted to assess its biosafety and effectiveness as an adhesive prosthetic device.

Results: During 35 days we observed no rabbit behavioral alteration, BP stability in the implantation site, good adhesion, and integration of the device with the surrounding tissue, and no adverse reactions.

Conclusions: BP could be used as an adhesive to secure the prostheses to tissues in abdominal wall prosthetic surgery, but large-size animal studies are needed.

Keywords: Buckypaper; Prosthetic abdominal wall surgery; Prosthesis fixation

Background

The adhesion of a surface on a biological tissue represents an important scientific and technological issue that excites the interest of many researchers. Different strategies were applied to prepare adhesive surfaces that can cling to organic substrates in physiological conditions [1,2]. Based on the well-known adhesiveness of foot pads of some animals like geckos, insects, spiders, or tree frogs [3,4], the research is generally but not exclusively focused on the achievement of surfaces composed of a structure of an array of nano-or micro-scale pillars [5,6] or microchannels, [7,8] possibly coated by highly hydrophilic molecules to increase the wet adhesion [9]. Compared to the flat unpatterned surface, animal-inspired structures show increased adhesion strength (scaling effect). Moreover, adhesion depends on geometrical surface features, including pillar or channel dimensions. Furthermore, density, [10,11] surface chemical composition, and experimental environment conditions (dry, wet,

or humidity-controlled) influence adhesivity [12,13]. Among various application fields, adhesives for biological surfaces can solve threatening biomedical problems whenever surgical prostheses should adhere to living tissues under physiological conditions, eliciting minimal adverse body reactions. Research in abdominal wall surgery is effervescent and aimed at creating innovative, light, patient-friendly, biocompatible prosthetic materials, free from the risk of complications, which reduce the duration of the operation and the costs of hospitalization. Suture line tension, mesh detachment, seroma formation, nerve trapping, hemorrhage, recurrence, and chronic postoperative pain represent the leading causes of complications [14-16]. Using human fibrin glue, although it has become a well-established surgical procedure, could potentially expose risks associated with the transmission of unknown diseases related to human blood-derived materials and high costs [17]. Moreover, using fibrin glue for mesh fixation increases the incidence of postoperative seroma [18]. The BP used in the research is nano-porous flexible felt, about 0.15-0.25 mm thick, composed of entangled unoriented oxidized multi-walled carbon nanotubes (MWCNTs). The BP adhesion was attributed to the suction of the fluid on the tissue into the material nano-pores. The negative capillarity drives the process that forces the compliant substrate to yield plastically and approach the BP surface [19]. Several papers and patents report the potential use of BP-based devices for applications in biomedical fields, [20-27] but more research needs on BP biocompatibility in mammals and surgical applications. Today, there are conflicting data concerning the safety and biocompatibility of CNTs [28]. Some researchers reported that CNTs show in vivo and in vitro cytotoxicity related to their acicular or fibrous particle shape or harmful impurities [29-31]. Other studies state that nanotubes aggregated as a thin sheet (as well as the BP) lose their toxicity. BP seems not toxic and does not affect the in vitro proliferation and viability of normal human arterial smooth muscle cells and human dermal fibroblasts [29-32]. In vivo experiments on the murine model showed that, although the BP induced a moderate inflammatory reaction, it had no mutagenic effects [30]. A cicatrization reaction with scar organization and fibrosis was recorded two weeks after BP implantation [32]. We report our experience assessing Buckypaper in a rabbit model to verify its applicability and safety as an adhesive for prostheses fixation to living tissues in abdominal wall surgery.

Methods

The Buckypaper (BP, Nano-lab inc.) is a nano-porous flexible felt pad, about 0.15-0.25 mm thick, composed of entangled and unoriented MWCNTs. "The treatment of MWCNTs with hydrochloric and nitric acid, suspension in water with a surfactant, and filtration on a membrane makes a free-standing continuous nanotube sheet characterized by a surface asymmetry due to the BP preparation and drying process used by the producer: one side is glossy, compact, and smooth (hereafter defined as BPs), while the other side is highly porous and rough (hereafter defined as BPr)" [19]. In vivo biocompatibility investigations were carried out by implanting BP in comparison with commercial Prolene Mesh (PR). We enrolled eight New Zealand female rabbits weighing about 3000 g (Harlan Laboratories). We anesthetized rabbits by intraperitoneal injection of xylazine 2 % (4.6 mg kg-1, Rompum, Bayer-Italia) and ketamine 10% (70 mg kg-1, Intervet Productions, Italy), maintained in a condition of spontaneous breathing during the operation and, finally, recovered by Antisedan injection (Pfizer). The surgical operation for the implantation started after 5 min from the general anesthesia. We implanted two rabbits with autoclaved (121°C for 21 min) buckypaper (BP) 2x2 cm^2 squared sample in a pocket created between the muscular fascia and large muscles of the abdominal wall (group A experimental BPR1-BPR2 Bp-subfascial). With the same method, two rabbits received a 2x2 cm^2 squared sterile Prolene mesh (PR, Ethicon) sample (group B PPR3 and PPR4 PP-subfascial). The wounds were then closed with absorbable stitches on the muscular fascia and not absorbable stitches on the cutaneous layer.

Moreover, two rabbits received a surgical incision on the abdominal midline deep into the cavity (group C experimental BPR5 and BPR6 BP-intraperitoneal). They received a 2x2 cm^2 BP squared sample inserted with the rough side facing the parietal peritoneum surface and the smooth surface facing the visceral peritoneum. The incision is closed with absorbable stitches on the fascia and not absorbable stitches on the cutaneous layer. All the animals received intra-operative and postoperative analgesia according to 86/609/EEC guidelines. Furthermore, two rabbits were not operated on but were observed (CTR7 and CTR8, group D, control not operated). The animals are housed in a temperature/humidity-controlled environment, 12-hour light/dark cycles, and have unrestricted access to water and standard rabbit food. The animals were monitored and controlled daily for thirty-five days after implantation, assessing their neurovegetative behavior (Irwin tests) [33] and the increasing curves of the rabbits' body weight (BW). Then, under general anesthesia, we sacrificed the animals to evaluate the adhesion of BP to the surrounding tissues, the local tissue reaction, histological sample trimming, and necroscopic and histopathological examinations. We excised wall portions with BP and surrounding fascial, dermal, and muscular tissues (to groups A, B, and C) for fixation in a 10% buffered formalin solution, alcohol dehydration, and impregnation with xylene and liquid paraffin at 58 °C embedding. The samples were cut by microtome into 3 mm thick sections, stained with Hematoxylin and Eosin (H&E), and analyzed by optical microscopy for histological observation.

Statistics: BW was measured weekly for all subjects randomized into four groups (Table 1). We calculated the BW mean values for each group (A, B, C, D) and growth curves calculated with Microsoft Excel 2010 (Figure 1). We performed experimentation following the policies and principles of standard laboratory animal

care and with the European Union guidelines (86/609/EEC) approved by the Italian Ministry of Health. The General Surgery Department Council (authorization n°159/20010-September 20, 2010) authorized the study.

groups	rabbit model	week 1	week 2	week 3	week 4	week 5
group A BPR1-BPR2 Bp-subfascial	BPR1	3022,1	3131,7	3231,1	3346,4	3458,8
	BPR2	3023	3128,5	3237,6	3343,6	3455,6
group B PPR3-PPR4 PP-subfascial	PRR3	3029	3172,9	3249,9	3371,4	3490
	PRR4	3033,1	3170,4	3251,1	3365,2	3486
group C BPR5-BPR6 BP-intraperitoneal	BPR5	3035	3133,4	3289,2	3473	3490,3
	BPR6	3031	3134,6	3286,8	3471,5	3488,1
group D CTR7-CTR8 control not operated	CTR7	3027,1	3198,4	3270,2	3379,1	3482,1
	CTR8	3025,3	3200,6	3268	3376,7	3501,7

Table 1: shows body weight monitoring in treated and control subjects.

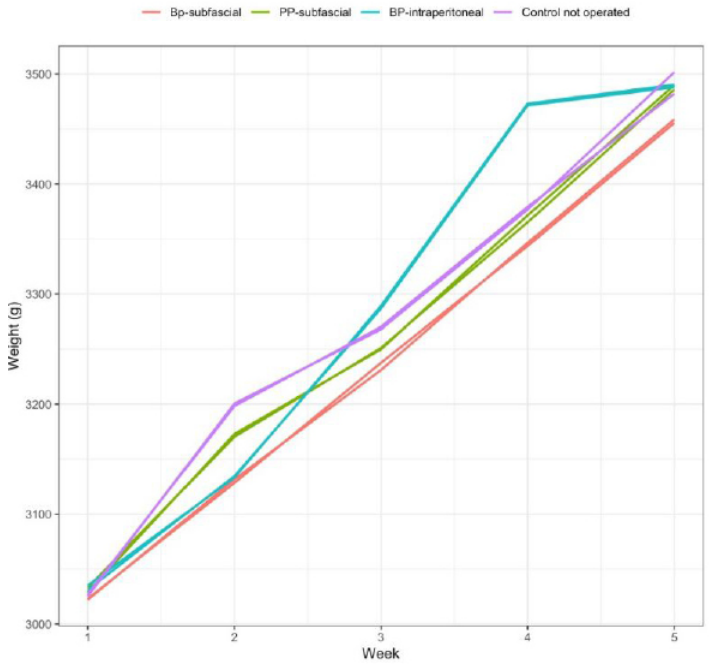

Figure 1: BP vs polypropylene comparison. Body weights were recorded before surgery and weekly after implantation until sacrifice at 5 weeks. Growth curves of body weight calculated on the mean value of each group of New Zealand rabbits. Analysis of Variance: F-statistic value = 0.2555; *p*-value = 1.31579; Anova test non-significant differences.

Results

Thorough thirty-five days after implantation, we evaluated the neurovegetative animal behavior, and the rabbits' body weight increased weekly, up the sacrifice. All subjects implanted with BP (BPR1, BPR2, BPR5, and BPR6), implanted with PR (PRR3 and PRR4), and not operated CTR7, and CTR8 did not show mortality, morbidity, or significant neurovegetative or behavioral differences, except a slight reduction of spontaneous activity immediately after surgery, probably due to the anesthesia.

Table 1 shows the body weight monitoring, and Figure 1 reports that operated and control animals followed the same weight increase up to the 35th day of the operation.

The mean growths observed in the four groups are:

group A experimental (BPR1-BPR2 Bp-subfascial): 434.65 gr

group B (PPR3-PPR4 PP-subfascial): 456.95 gr

group C (experimental BPR5-BPR6 BP-intraperitoneal): 456.2 gr

group D (CTR7-CTR8 control not operated): 465.7 gr

After sacrifice, we studied BP's adhesion to the surrounding tissues, the local tissue reaction, the cicatrization, and the necroscopy and histopathological examinations. We did not observe adverse reactions from the tissue surrounding the implant. Histopathology showed an excellent integration with the surrounding tissues of the BP rough surface (BPr) implanted in the muscular pocket in group A. No prosthesis folding, displacement, shrinkage, or seroma are observed in BP-implanted rabbits. Macroscopically, the BPs implanted into the subfascial muscular pocket (BPR1 and BPR2) showed a weak integration with the adjacent tissues that maintained a physiologic color and consistency. Figure 2 shows black BP debris less than 10 microns in size, surrounded by a layer of epithelioid cells, giant cells, and some at 400x. We observed in the cytoplasm of some giant cell inclusions of tiny fragments of BP. (Figure 3). (A-D) Photomicrographs of H&E stained tissues-BP interface from BPR2. (A) BPs side (x 50). (B) BPs side (x 200). (C) BPr side (x 50). (D) BPr side (x 200).

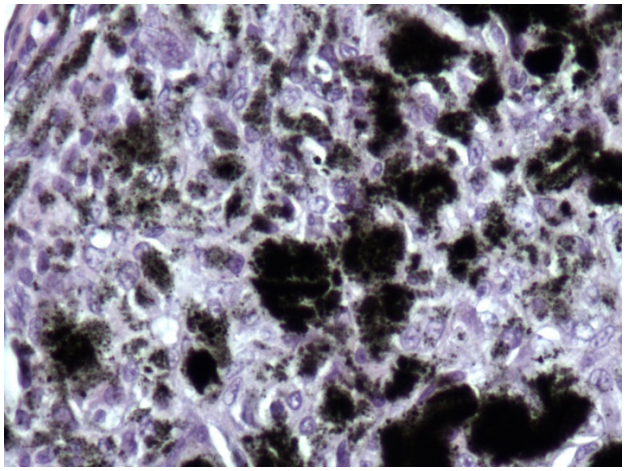

Figure 2: Study of BP implantation in rabbit dermis 35 days after implantation at 400x. The figure shows black BP debris less than 10 microns in size, surrounded by a layer of epithelioid cells, giant cells and some fibroblasts. In the cytoplasm of some giant cell cells, inclusions of tiny fragments of BP are observed. In conclusion, contrary to what was supposed, BP fragments are phagocytosed and reabsorbed by giant and epithelioid cells.

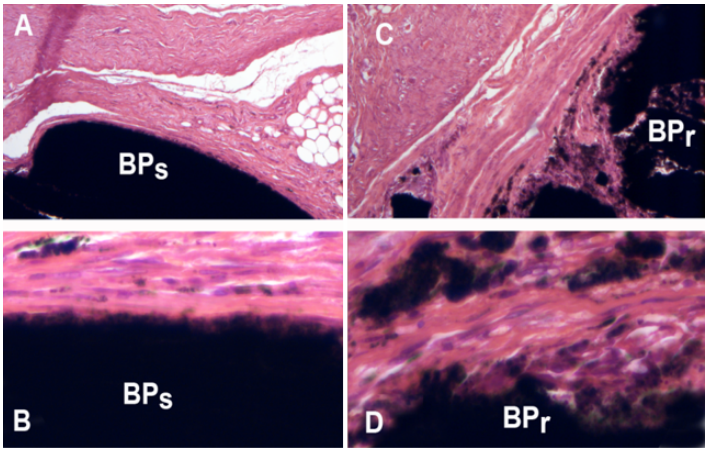

Figure 3: (A-D) Photomicrographs of H&E stained tissues-BP interface from BPR2. (A) BPs side (50x). (B) BPs side (200x). (C) BPr side (50x). (D) BPr side (200x). Some BP micro-sized debris was phagocytized by macrophages (400x enlargements in Figure 2).

Besides a small fragmentation at the BPs and muscular tissue interface, the images show the deposition of loose fibrous tissue, consisting of fibroblasts and collagen fibers, indicative of a weak inflammation reaction. On the other side, the BPr surface firmly adhered to the muscular tissue. The micrograph of Figure 3 C and D (magnification 50x and 200x, respectively) displays a higher BP fragmentation at the BPr at the tissue interface and moderate chronic inflammation reaction, as assessed by the presence of fibroblasts, collagen fibers, and macrophages. Neither multinucleated foreign body giant cells nor neutrophil granulocytes were observed, indicative of abscess formation. Macrophages phagocytized the micro-sized debris from the partial fragmentation of implanted BP sample (Figure 2). The necroscopic examination of PRR3, and PRR4, revealed that the implanted PR favored the cicatrization process around the mesh, as widely described in the literature.[35,36] However, unlike BPR1 and BPR2, the necroscopy evidenced an opaque and thick peritoneal surface under the implantation site in PRR3 and PRR4.

Necroscopy on BPR5 and BPR6 showed that the BPs surface exposed to the gut contained in the abdominal cavity did not develop inflammation, adhesions to the omentum, or intestinal loops. Moreover, no thick fibrous capsule formation occurred, and the BPs surface appeared loosely adherent, glossy, and translucent. The surrounding peritoneal surface had a physiologic color and consistency. On the contrary, the BPr surface showed strong adhesion and integration with the peritoneal surface. Furthermore, BPs show a scarce interaction facing the visceral peritoneal

surfaces in BPR5 and BPR6, so the soft adherences were easily separated from the gut, and reperitonealization occurred. A monocellular mesothelial carpet covered BP's smooth surface, similarly prostheses for intraperitoneal use.

Discussion

The monitoring of the body weight shown in Figure 1 shows that all the operated animals and the non-operated control subjects follow the same growth curve without marked differences because the operated animals well tolerate the implanted BP and PR. Moreover, there are no differences between group A and group B. In other words, the implanted subjects in group A tolerates BP well, as group B tolerates the PR. "BP rapidly absorbs high amounts of water, up to 4 times its dry weight." [19] "The biological fluid on animal tissues' surface is rapidly absorbed by BP because of it's the negative capillary pressure. The suction pushes the soft and compliant substrate into closer contact with the relatively hard BPr surface, favoring the increase of actual interface area and the reduction of the distance between the two adhering materials. The possibility of the compliant substrate to conform on the rigid BP surface asperity, besides the adhesion, also increases the interface's shear resistance by forming mechanical interlocking, active mainly on the BPr surface" [19]. For these reasons, we suppose that BP could be interesting for surgical application as adhesive tape for prostheses fixation in the abdominal wall reconstructive surgery such as the groin prosthetic surgery, the incisional hernias repairing, the Grynfelt's lumbar quadrangle, and Pettit's triangle hernias repair, the abdominal wall disaster, the diaphragm traumatic rupture, the Delorme's surgical procedure of the prolapsed colon, and the cystocele caused by urinary bladder prolapse. It is relevant that peeling strengths found in BP [19] resulted to be higher than those recorded by Jacob, et al. [34] or by Eriksen et al. [35] on different commercial prosthetic meshes implanted in pig models, with or without fibrin glue, respectively, and by Mahdavi et al. on nanopatterned poly (glycerol sebacate acrylate) surface implanted in rats [1]. "To assess the BP behavior variation after its first placement on a living substrate, simulating a possible erroneous positioning during surgery, a second run of the peeling test was performed. " [19] "In the second experiment the adhesion strength decreased, probably because of the liquid absorbed by BP during the previous run. The filling of BP pores by the biological fluid can decrease the negative capillary pressure and, hence, the adhesion. The observed behavior can also be related to the residual biological tissue on the BP after the first experiment, which can obstruct the pores, or to the drying of the tissue surface that occurred during the first experiment. However, a good adhesion persists" [19].

Necroscopic and histological investigations enlightened that, at the sacrifice, the BP elicited minimal adverse tissue response, as assessed by the absence of granulocytes, neutrophils, and giant cells. Moreover, the rough, porous surface favored strong and stable integration with the surrounding tissues, as evidenced by necroscopic examination. The scar tissue growth around the implanted BP ensures good positional stability. "The peeling test is meaningful for the BP rough surface (BPr) behavior" [19]. "Peeling, and shear tests measured the force necessary to detach the commercial prosthetic meshes and BP tape from the biological substrate" [19]. Those experiments provide essential information about BP adhesivity on living tissues and, hence, on BP's stability in the implantation site and its capacity to shorten the surgical procedure duration. We did not observe adhesions between the BPs surface exposed to the peritoneal cavity and the greater omentum or bowel loops. The BPs surface facing the abdominal cavity explanted from BPR5 is covered by a thin protein layer which is also covered by a monolayer of peritoneal polygonal mesothelial cells, and some fibrocytes grew in some sample regions. This coverage prevents gut adhesions. Figure 2 shows black BP debris less than 10 microns in size, surrounded by a layer of epithelioid cells, and in the cytoplasm of some giant cell inclusions of tiny fragments of BP. Contrary to what was supposed, BP fragments are phagocytosed and reabsorbed by giant and epithelioid cells. Micrometric carbon nanotube aggregates, deriving from the BP surface fragmentation of the implanted BP sample (Figure 3 D), mainly on the rough surface, were phagocytized by macrophages. Macrophages engulf microscopic-sized debris, which means that the BP is absorbed and metabolized. It is crucial to understand if the circulating fragments can pass into the urine and metabolization in light of BP applications in surgery. To definitively assess BP debris' possible toxicity, confinement, metabolism, and accumulation or excretion mechanism and the BP biosafety and biocompatibility, a broader investigation on larger quadrupedal animals and over a longer monitoring time is necessary to follow the destiny of such BP debris to assess their possible local or systemic toxicity and their excretion mechanism. The BP application as an adhesive tape is supposed for prostheses fixation in abdominal wall surgery.

Limitations

The main limitation is the small sample size of subjects which didn't allow us to perform the inferential statistics. We intend this study as a pilot study to test whether BP is safe and reliable in vivo and whether it could find application in a particular type of surgery that could benefit from a technological advance as justification for the scarcity of resources. We are well aware that the number of subjects used is small (8 subjects divided into four groups), and it was impossible to perform an Analysis of Variance, F-statistic value, and p-value, and the ANOVA test, even if no differences among the four curves were enlightened. Moreover, the duration of the follow-up is only five weeks, and the absence of comorbidities (often affecting human patients). The community knowns data on the behavior of implanted PR (polypropylene is

the current standard used in prosthetic surgery used for comparison with BP) in the human and animal model [36-40].

Conclusions

This experimentation helps us to understand that BP shows attractive abdominal wall prosthetic surgery qualities, such as adhesiveness for prosthesis fixation, biocompatibility, and resorbability. BP does not cause seromas or hematomas, is simple to use, can be detached and repositioned without causing injury to the fascia and muscles, and could shorten surgical times. The rough side of BP shows a higher shear and peeling adhesion strength. The smooth side of BP shows poor shear or peeling adhesion strength and could be helpful to face viscera and omentum in the abdominal cavity with scarcely adherent adhesions with the greater omentum or bowel loops. Moreover, BP elicits minimal adverse tissue response. Macrophages phagocytize micrometric BP debris, but assessing their possible toxicity, confinement, accumulation, and excretion mechanism need extensive studies on big-size animals.

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