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Effects of Different Capsule Types for Oral Application of Active Substances – Investigations with Organ-Specific Cells

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Abbrevations:

HPMC: Hydroxypropyl methylcellulose; PEG: Polyethylene glycol; $HPMC_{pr}$: Premium HPMC; polyethylene glycol and carrageenan free capsules; $HPMC_{st}$: Market standard HPMC capsules; PBS+: Phosphate-buffered saline with calcium and magnesium

1. Abstract

1.1. Background: A capsule is a solid dosage form that delivers a defined dose of an active ingredient. Most capsules are made for oral application by mouth with subsequent swallowing. Capsule materials for the shell are not only of importance for the release of active ingredients, but should also be without any impact on the digestive tract and the active ingredient itself. Some capsule materials have been shown to possess unwanted effects after digestion.

1.2. Materials and Methods: By using different organ-specific cell cultures such as connective tissue fibroblasts, intestinal epithelial cells and functional neutrophils, we examined three different commercially available and commonly used capsule types for unwanted side-effects. We examined (i) hydroxypropyl methylcellulose (HPMC) capsules (market standard), (ii) Pullulan capsules and (iii) HPMC capsules free of polyethylene glycol and carrageenan. We examined antioxidant and acute cytotoxic effects as well as the effect on wound healing process, basal cell metabolism and proinflammatory potential of aqueous capsule solutions.

1.3. Results: The three capsule types reacted very different in the tests, but with the HPMC capsules free of polyethylene glycol and carrageenan performing in most tests very well. This was particularly evident by the minor effects on cell vitality, cell regenerative potential and proinflammatory potential when compared to HPMC (market standard) or Pullulan capsules. From the results of the study presented here, the use of more expensive HPMC capsules free of polyethylene glycol and carrageenan for application of orally administered active ingredients can be highly recommended.

2. Introduction

A capsule is a solid dosage form that delivers a defined dose of an active ingredient. Most capsules are made for oral application by mouth with subsequent swallowing. This dosage form can ensure both a correct dosage and a simplified regular intake. Capsules consist of a capsule shell and a filling. Both the shell and the contents can be made resistant against acidic stomach juice with coatings or other techniques, so that the active ingredients are only released in the intestine. While hard capsules are preferably filled with solid contents such as powders, granules, mini tablets or even smaller capsules, soft capsules are generally filled with liquid or pasty contents.

The classic capsule shell is made of gelatin which is derived from collagen, a wide-spread natural structural protein, predominantly found in the connective tissues of animals [1]. The main component of gelatin is denatured (hydrolyzed) collagen. Gelatin is commonly used as a food ingredient, for medications, drug and vitamin capsules, cosmetics and many others [2]. Besides gelatin, there are some other compounds used alone or in varying combinations for the capsule shell: (1) Hydroxypropyl methylcellulose (HPMC) is a synthetic mod-

ification of the natural polymer cellulose, that has been produced by complex chemical processes. This form of the capsule shell is used very widely for application of all available forms for nutritional supplements and is also offered as a vegan capsule. It is largely replacing the animal-derived gelatin in conventional two-piece capsules [3]. It is used in the food industry as a multipurpose food ingredient. HPMC is approved by FDA as both a direct and an indirect food additive, and is approved for use as a food additive by the EU [4]. However, the air barrier protection is significantly lower than for gelatin and pullulan capsules. (2) Pullulan is a water-soluble glucan gum produced aerobically by growing a yeast-like fungus *Aureobasidium pullulans*. Only poly-maltotriose, guar gum, algae extract, acetic acid and purified water are used in the manufacturing of pullulan capsules [5]. It has a wide range of commercial and industrial applications in many fields like food science, health care, pharmacy and many others. A pullulan capsule shell has very good air barrier properties. (3) Carrageenan is a sulphated linear polysaccharide of D-galactose and 3,6-anhydro-D-galactose obtained by extraction of certain red seaweeds. The polysaccharide has residues that can bind ions such as sodium, potassium and calcium. Carrageenan is found in many foods [6]. It is primarily used as a stabilizer and thickener in food, but also has a variety of applications in drug delivery [7]. Carrageenan is the most controversially discussed capsule shell material ranging from harmless [8] to carcinogenic [9] or being an activator of the inflammatory cascade in human intestine [10].

Prompted by this background we investigated the cellular effects of three different capsule shell materials on organ-specific cultured cells by using test concentrations which are similar to real life conditions.

3. Material and Methods

3.1. Capsule Types and Test Concentrations

We examined the following 3 different capsule types: (1) Market standard HPMC capsules, named here as "$HPMC_{st}$"; (2) Pullulan capsules, named here as "Pullulan" and (3) Premium HPMC: Polyethylene glycol (PEG) and carrageen free capsules, named here as "$HPMC_{pr}$". The capsule weight of the 3 different capsule types tested here was 1,520 mg for $HPMC_{st}$, 1,990 mg for pullulan and 2,450 mg for $HPMC_{pr}$ (weight for 20 capsules each). Independent from capsule weight, a maximum daily intake of 20 capsules was assumed to enter 1,000 ml of intestinal fluid. This is equivalent to a test concentration of 2,000 µg/ml. Thus, basic 10x stock solutions were prepared by dissolving 40 capsules in 100 ml of phosphate-buffered saline with calcium and magnesium (PBS+), pH 7.4. Further dilutions were made in PBS+. The test concentrations (dilutions of the basic stock solutions in brackets) in the tests were: 40 µg/ml (1:100), 80 µg/ml (1:50), 200 µg/ml (1:20), 400 µg/ml (1:10), 800 µg/ml (1:5), and 2,000 µg/ml (1:1).

3.2. Examination of the Antioxidant Effect Using a Cell-Free Test System

In this cell-free test system, it was tested whether the various solubilized capsule types are capable of inactivating free oxygen radicals (antioxidant effect). The different dilutions in distilled water were subjected to a potassium superoxide solution (1 mg KO_2/ml distilled water). The superoxide anion radicals present in the reaction mixture caused a cleavage and a change in optical density (= color) of the water-soluble tetrazolium dye WST-1 (Sigma-Aldrich, Deisenhofen, Germany), which was also added to the mixture [11, 12]. The optical density was recorded as a differential measurement ΔOD = 450 minus 690 nm using an Elisareader (BioTek ELx808 with software Gen 5 Version 3.00) for the time interval 0-30 min and analyzed by Microsoft Excel.

3.3. Organ-Specific Cells Used in This Study

The following cell lines were used: (1) Intestinal epithelial cells IPEC-J2 (ACC-701; normal epithelial cells from the small intestine (jejunum) from pigs) were used in the sub cultivation stages (passages) 16 to 18. Cells were routinely cultivated in DMEM with 4.5 g/l glucose and Ham's F12 (1:1), 10% growth mixture and 0.5% gentamycin. (2) Connective tissue fibroblasts L-929 (ACC-2; normal connective tissue fibroblasts from mouse) were used in passages 98 to 100. Cells were routinely cultivated in RPMI 1640 with 10% growth mixture and 0.5% gentamycin. (3) Human promyelocytes HL-60 (ACC-3; acute myeloid leukemia cells used for induction of differentiation studies). Cells were routinely cultivated as suspension cultures in RPMI 1640 with 10% growth mixture and 0.5% gentamycin.

All cell lines were from Leibniz Institute, German Collection for Microorganisms and Cell Cultures, Braunschweig, Germany, and incubated in an incubator at 37°C and an atmosphere of 5% CO_2 and 95% air at almost 100% atmospheric humidity.

3.4. Examination of cytotoxicity (IPEC-J2 and L-929)

The tests were conducted according to EN ISO 10993-5. Cells were seeded from 80-90% confluent mass cultures into 96-well culture plates (200 µl culture medium/well) at a density of 5,000 cells/well and incubated for 24 hours until the cells were completely attached and spread. Culture medium was exchanged and the different capsule dilutions and PBS+ as the reagent control were added. After three days of continuous exposure, culture medium was aspirated and 180 µl of fresh culture medium and 20 µl of XTT (Xenometrix, Allschwil, Switzerland) were added to each well for examination of cell vitality. XTT is the sodium salt of 2,3-bis [2-methoxy-4-nitro-5-sulfopheny]-2H-tetrazolium-5-carboxyanilide and has a yellowish color. Mitochondrial dehydrogenases of living cells cleave the tetrazolium ring of XTT and orange-colored, water-soluble formazan crystals are formed. The intensity of the resulting color of the reaction solution is proportional to cell vitality [13, 14]. Finally, the optical density of each well was measured after 0 min and after 120 min at 37 °C as a differential measurement ΔOD = 450 minus 690 nm using an Elisareader (BioTek ELx808 with software Gen 5 Version 3.00) and compared with the corresponding reagent control.

Moreover, cell cultures were screened morphologically for signs of

visible toxic effects such as rounding, detachment or vacuolization.

3.5. Examination of Cell Regeneration/Wound Healing (IP-EC-J2)

In the test system used here, especially the proliferative phase which is characterized by the occurrence of cell migration and cell proliferation was simulated [15, 16]. Intestinal epithelial cells were seeded at a density of 50,000 cells/ml in the three individual compartments of a culture-insert 3 well made of silicone (ibidi, Planegg, Germany). The compartments of the inserts are separated from each other by a 500 µm thick silicone frame. Due to its special adhesion area, an insert adheres firmly to the bottom of a culture dish and results in a defined cell-free area. After reaching confluency within 48 hours after seeding, the inserts were carefully removed using sterile tweezers. Then, the cells were able to migrate into the resulting cell-free areas and closing them by an increased proliferative activity. After 8 hours the cells were fixed with methanol p.a., stained with Giemsa's azure-eosin-methylene blue solution at pH 6.5 and the width of the remaining cell-free space was measured. Only one concentration was tested in quadruplicate cultures (1,600 µg/ml; dilution of 1: 2.5).

3.6. Examination of Proinflammatory Potential (Functional Neutrophils)

The infiltration of a tissue by neutrophils is a hallmark of acute and chronic inflammatory disorders, among them is the gastrointestinal tract [17]. By cultivating promyelocytes in a medium containing 1.5% dimethyl sulfoxide for 6 days [18], cells were differentiated into functional neutrophils [19, 20]. These are cells possess the properties of inflammation-mediating cells in vivo and are also able to generate reactive superoxide anion radicals [21]. By doing so, they can prolongate or even complicate the wound healing process within the gastrointestinal tract [22].

The resulting functional neutrophils were collected by centrifugation (190 x g for 6 min) and washed twice with PBS+. Finally, they were resuspended in PBS+ containing 10 mM glucose and pipetted into the reaction mixture. The reaction mixture consisted of a phorbol ester (phorbol-12-myristate-13-acetate; Sigma-Aldrich, Deisenhofen, Germany) to induce the generation of superoxide anion radicals by the cells and WST-1, which was cleaved by the radicals present in the reaction mixture. The amount of oxygen radicals was directly related to the color change, i.e. a proinflammatory effect was noticeable by a stronger color change per time. The optical density was recorded at different time points as a differential measurement ΔOD = 450 minus 690 nm as already described here. Without the addition of the phorbol ester to the reaction mixture to induce an oxidative burst, we also examined the basal cell metabolism at different capsule test concentrations.

3.7. Statistical Analysis

The two-tailed Wilcoxon-Mann-Whitney test was used for statistical analysis of the measurement data. Significance was achieved at p values ≤ 0.05.

4. Results

4.1. pH values

All three capsule types were checked for pH after 40 capsules had been dissolved in 100 ml of distilled water. All three aqueous capsule solutions reacted slightly acidic. The pH values were as follows: $HPMC_{st}$ capsules: pH 6.15; pullulan capsules: pH 5.93; $HPMC_{pr}$ capsules: pH 6.53. However, cell culture medium containing the capsule dilutions did not show any pH shifts.

4.2. Antioxidant Effect

As shown in (Figure 1), all dilutions of the different capsule stock solutions showed no significant deviation from the reagent control. Only $HPMC_{pr}$ was slightly antioxidant at all dilutions.

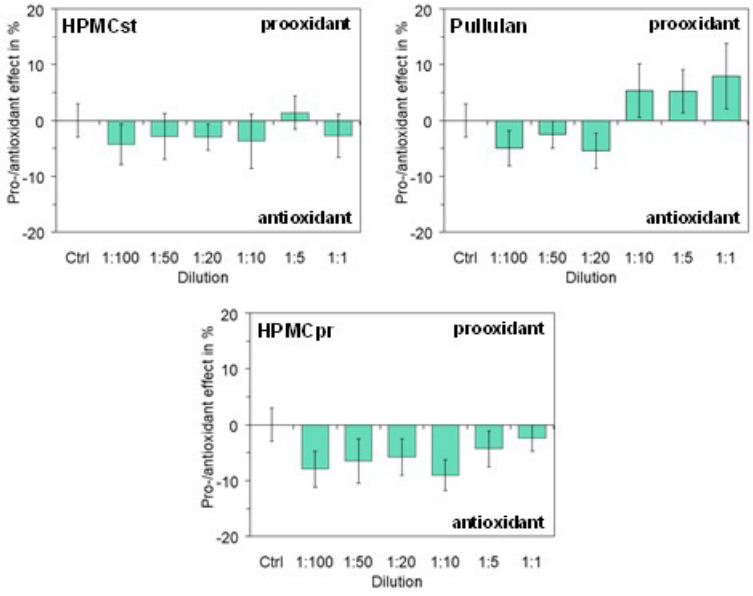

Figure 1: Pro- and antioxidant effect of different capsule dilutions using a cell-free test system. The control is set as "0 %". Only HPMCpr was slightly antioxidant in all dilutions. Data represent mean value ± standard deviation of 3 parallel experiments. HPMCst = market standard HPMC capsules; HPMCpr = polyethylene glycol and carrageen free capsules.

4.3. Cytotoxic Effect

After three days of continuous exposure, all three capsule types showed no cytotoxic effect at all dilutions tested when using intestinal epithelial cells (Figure 2). For all capsule types there was even a slight increase in cell viability in comparison to the reagent control which became statistically significant for pullulan at a dilution of 1:1 ($p \leq 0.05$). There was no change in epithelial cell morphology.

Exposure of more sensitive connective tissue fibroblasts for three days showed different effects for all three capsule types (Figure 3). For $HPMC_{st}$, cell vitality was significantly reduced in comparison to the reagent control by a maximum of 10.2 ± 3.9 % (mean value ± standard deviation; $p \leq 0.05$) at a dilution of 1:1. For pullulan, the dilutions of 1:5 and 1:1 showed a significant reduction in cell vitality ($p \leq 0.05$) in the same range as for $HPMC_{st}$. Only for $HPMC_{pr}$ dilutions there was no significant difference to the reagent control.

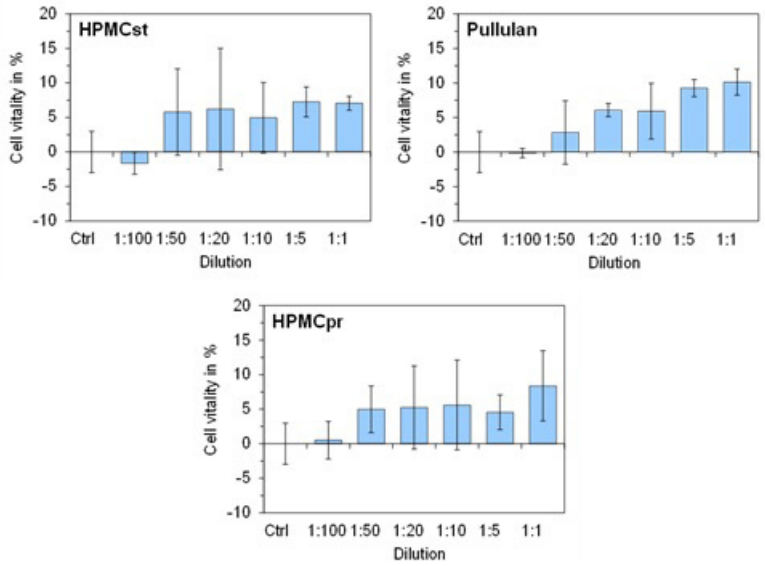

Figure 2: Effect of different capsule dilutions after 3 days of incubation on the vitality of cultivated intestinal cells (IPEC-J2). No cytotoxic effect can be observed. The control is set as "0 %". Data represent mean value ± standard deviation of 3 parallel experiments. HPMCst = market standard HPMC capsules; HPMCpr = polyethylene glycol and carrageen free capsules.

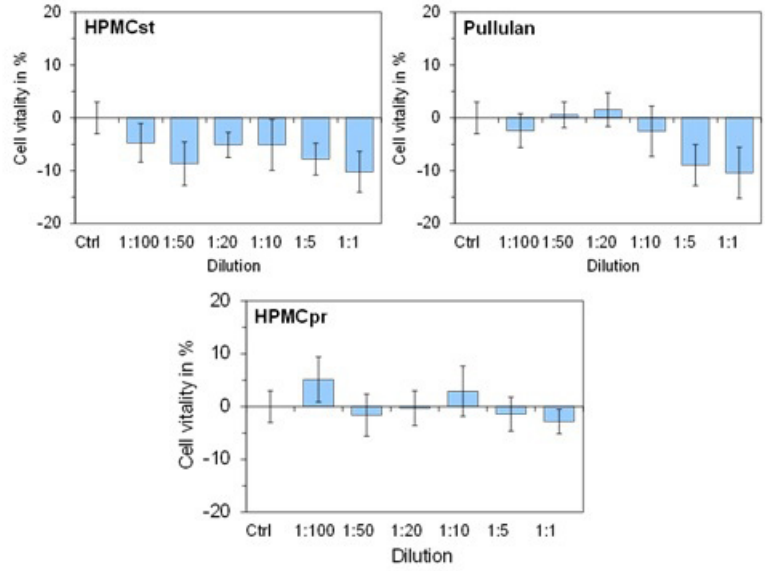

Figure 3: Effect of different capsule dilutions after 3 days of incubation on the vitality of cultivated connective tissue fibroblasts (L-929). Note that only HPMCst and Pullulan, but not HPMCpr, are slightly cytotoxic at a dilution of 1:1. The control is set as "0 %". Data represent mean value ± standard deviation of 3 parallel experiments. HPMCst = market standard HPMC capsules; HPMCpr = polyethylene glycol and carrageen free capsules.

4.4. Effect on Wound Healing Process of Intestinal Epithelial Cells

As can be seen from the micrographs in Figure 4, the colonization of the cell-free space was quite inhomogeneous in each sample due to the migration pattern of intestinal epithelial cells. Therefore, four experiments had to be conducted, each with 12 measurements of the cell-free space. In comparison to the control we observed a reduced closure of the cell-free space for $HPMC_{st}$ by 29.7 ± 11.0 % and by 32.7 ± 9.2 % for pullulan (mean value ± standard error of the mean). Both values were significantly different from the control ($p \leq 0.05$). Only for $HPMC_{pr}$ the reduction was 8.9 ± 9.3 % (mean ± standard error of the mean) and did not differ from the control.

4.5. Basal Cell Metabolism of Functional Neutrophils

As shown in (Figure 5), the three capsule type dilutions tested had completely different effects on the basal cell metabolism of functional neutrophils. $HPMC_{st}$ was more or less indifferent in comparison to the control, whereas pullulan caused a significant reduction in cell metabolism at all dilutions ($p \leq 0.05$). In contrast, $HPMC_{pr}$ caused a significant and dose-dependent stimulation of cell metabolism with a maximum stimulation of 125% at a dilution of 1:5 in comparison to the control ($p \leq 0.01$).

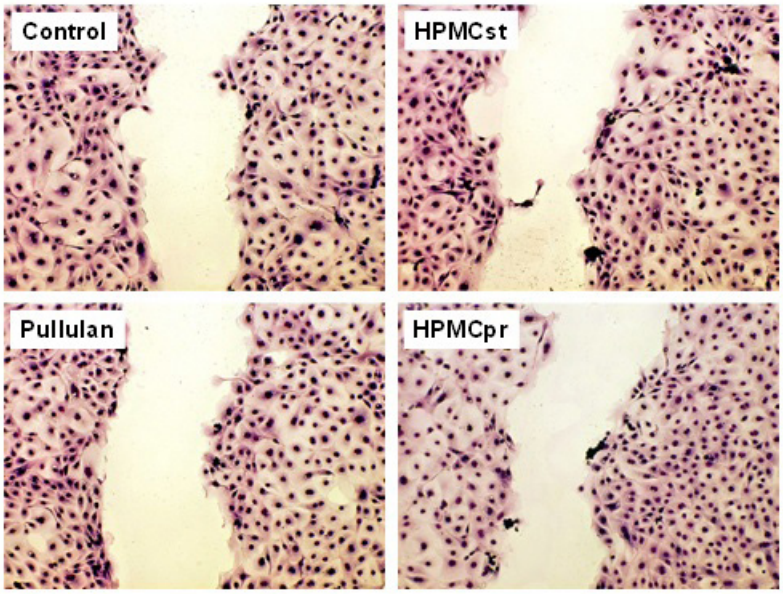

Figure 4: Wound healing process of intestinal epithelial cells (IPEC-J2) by colonization of a cell-free space within 24 hours at a dilution of 1:2.5 (= 1,600 µg/ml) for the different capsule solutions. Fixed and stained samples were photographed using an Olympus IX-50 inverted microscope with an Olympus 10x planachromate and an Olympus E-10 digital camera at 4-megapixel resolution. HPMCst = market standard HPMC capsules; HPMCpr = polyethylene glycol and carrageen free capsules.

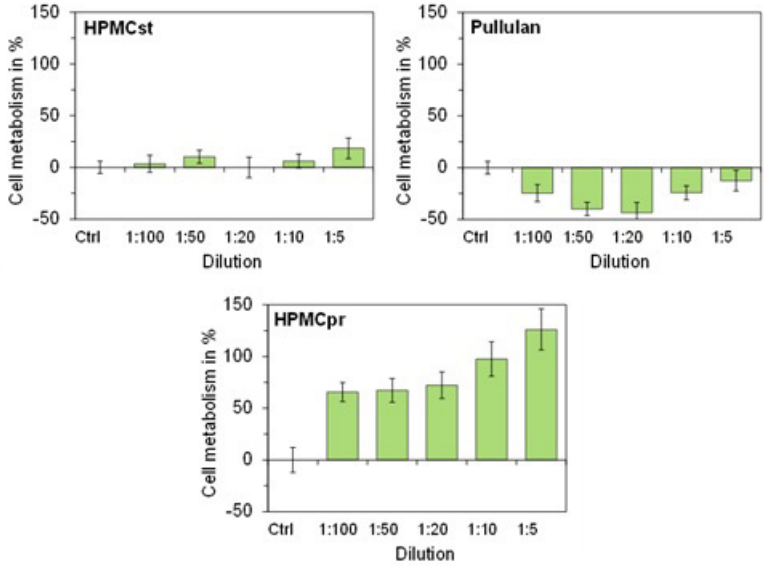

Figure 5: Effect of different capsule dilutions on basal cell metabolism of functional neutrophils. Note that the effect of HPMCst is similar to the control, whereas pullulan significantly reduces basal cell metabolism and HPMCpr promotes it in a dose-dependent manner. The control is set as "0 %". Data represent mean value ± standard deviation of 3 parallel experiments. HPMCst = market standard HPMC capsules; HPMCpr = polyethylene glycol and carrageen free capsules.

4.6. Proinflammatory Potential

The examination of an unwanted proinflammatory effect with functional neutrophils by superoxide anion radical generation after stimulation by an phorbol ester showed a dose-dependent increase of more than 75% for $HPMC_{st}$ and approximately 70% for pullulan (Figure 6). For $HPMC_{st}$ the proinflammatory effect was statistically significant at all capsule dilutions ($p \leq 0.01$) and for pullulan only at dilutions of 1:10 and 1:5 ($p \leq 0.01$). In contrast, $HPMC_{pr}$ had a maximum proinflammatory effect of only 20% which was statistically significant at dilutions of 1:10 and 1:5 ($p \leq 0.05$).

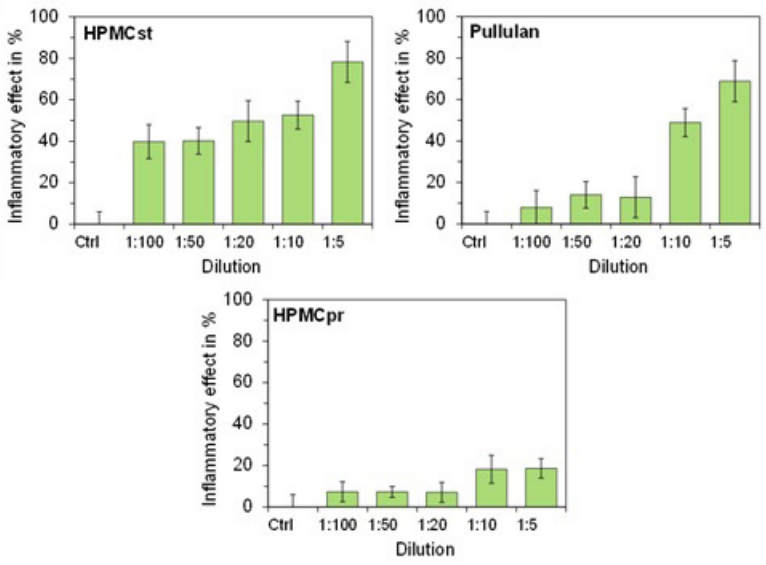

Figure 6: Effect of different capsule dilutions on the proinflammatory response of functional neutrophils by the generation of superoxide anion radicals. Note that only HPMCst and pullulan cause a strong and dose-dependent increase in radical generation, whereas the proinflammatory effect of HPMCpr is considerably lower. The control is set as "0 %". Data represent mean value ± standard deviation of 3 parallel experiments. HPMCst = market standard HPMC capsules; HPMCpr = polyethylene glycol and carrageen free capsules.

5. Discussion

When looking at the present study, most of the results within the range of a normal capsule intake did not differ from the controls. When looking at the maximum test concentrations we found some cytotoxic effects. Any stimulatory effects such as an increased cell metabolism might be related to the different carbohydrate content of the materials which is brought into the culture medium by dissolving the capsules.

The most striking differences between the three capsule materials were observed in the case of (i) cell regeneration of intestinal epithelial cells and (ii) proinflammatory potential of the capsule materials with functional neutrophils. Both features are closely related to each other, because intestinal integrity and regeneration is also decreased by acute and chronic inflammatory disorders which is triggered by the infiltration of neutrophils into the gastrointestinal tract [17, 22].

In both test series, only $HPMC_{pr}$ capsules, which are free of polyethylene glycol and carrageenan, have passed the tests without any significant unwanted effects at test concentrations representing a normal use. Then, $HPMC_{pr}$ capsules did not reduce intestinal cell regeneration and did not induce a proinflammatory response of functional neutrophils.

From our results it can be concluded that unwanted side effects of capsule materials might be due to their content of carrageenan which is still a controversially discussed capsule shell material. Carrageenan has been stated as harmless [8] or carcinogenic [9]. However, the most relevant and unwanted property of carrageenan in relation to our study is an activation of the inflammatory cascade in human intestine as demonstrated in a recent study [10]. Already 50 years ago, Watt and Marcus first observed ulcerations of the large intestine in response to carrageenan exposure in experimental animals [23]. In numerous animal studies the finding of a disruption of the intestinal epithelial barrier, inhibition of proteins that provide protection against microorganisms, and stimulation of the elaboration of proinflammatory cytokines has been reported for review, see [24-26]. However, the specific mechanism(s) by which carrageenan induces inflammation in experimental animal models are not clearly defined yet. In addition, there are also reviews on in vitro studies of carrageenan effects on cultured intestinal cells or intestinal models [27].

In conclusion, the use of more expensive HPMC capsules free of polyethylene glycol and carrageenan for application of orally administered active ingredients can be recommended.

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