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EFEITO DA IDADE NA VIABILIDADE DE EXPLANTES INTESTINAIS: UMA ABORDAGEM HISTOLÓGICA E ULTRAESTRUTURAL

(Effect of age on the viability of pig intestinal explants: a histological and ultrastructural approach)

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RESUMO: Os explantes intestinais são utilizados como modelo para estudos toxicológicos e patológicos, mas a manutenção da sua viabilidade por longos períodos de incubação ainda se mostra como um desafio. O objetivo desse estudo foi avaliar o efeito da idade na viabilidade de explantes intestinais. Explantes do jejuno foram obtidos de suínos de 24 e 42 dias de idade. Os explantes foram incubados por duas ou quatro horas e foram processados para análise histológica. Os explantes dos suínos mais jovens apresentaram menores alterações histológicas, avaliados por escore tecidual e avaliação morfométrica. Os explantes dos suínos de 24 dias de idade foram incubados por quatro horas e submetidos a análise ultra estrutural para avaliação da integridade celular. A membrana plasmática, núcleos e organelas dos enterócitos se mantiveram bem preservadas e foi possível observar a viabilidade funcional do tecido devido a presença de vesículas de endocitose. A morfologia intestinal de explantes incubados dos animais mais jovens foi melhor preservada que aquelas de explantes dos animais mais velhos.

Palavras-chave: ex vivo, intestino, jejuno, suíno

ABSTRACT: Intestinal explants are a useful model for toxicological and pathological studies, but the maintenance of tissue viability for longer periods of incubation remains challenging. The aim of this study was to evaluate the effect of age on the viability of intestinal explants. Jejunal explants were obtained from 24- and 42-day-old piglets. The explants were incubated for two or four hours and were then processed for histological analysis. The explants from younger pigs showed minor histological changes, as assessed by histological and morphometrical scores. Explants from 24-day-old pigs were incubated for four hours and subjected to ultrastructural analysis to assess cell integrity. The plasma membranes, nuclei and organelles of enterocytes were well preserved, and adsorptive endocytosis was observed. In conclusion, the data indicate that the age of the donor influences explant viability. The intestinal morphology of incubated explants from young donors was better preserved than that of explants from older piglets.

Key Words: ex vivo; intestine; jejune; swine

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INTRODUÇÃO

The gastrointestinal (GI) tract is a complex organ consisting of multiple layers and cell types with different functions, such as the absorption of nutrients and the maintenance of a physical and immunological barrier against pathogens. Diseases of the GI tract represent some of the major problems in human and animal health (Menin *et al.,* 2008), and the establishment of suitable models to investigate the morphological or pathological aspects of these diseases has attracted increasing interest.

The complexity of intestinal structures makes investigations of morphological or pathological aspects using cell culture models difficult (Randall *et al.,* 2011). However, the use of an *ex vivo* approach, such as intestinal explants, is suitable to mimic the GI tract. Compared to *in vivo* models, cultured explants allow greater environmental control, the direct application of predefined doses onto target organs and the comparison of treated and control samples from a single donor with more reliable results (Randall *et al.,* 2011). Furthermore, the explant model permits the preparation of large numbers of explants from a single animal, reducing the number of experimental animals needed (Kolf-Clauw, 2009)

In pigs, the explant model has been described in studies of bacterial adhesion (Zhu *et al.,* 1995), viral replication (Poucke *et al.,* 2010) and mycotoxin toxicity (Kolf-Clauw, 2009). In this context, pigs are similar to humans in terms of digestive anatomy and physiology and therefore constitute a suitable model for the study of gastrointestinal changes (Swindle, 1998; Guilloteau *et al.,* 2010).

The scarcity of studies using intestinal explants could be related to the difficulty of maintaining tissue viability. Some studies have reported an association between the age of the donor and tissue viability, which may be due to the increased resistance of young tissue to hypoxia (Trowell, 1959; Kolf-Clauw *et al.,* 2009).

The explant model could be very useful for morphological and toxicological studies. However, few studies have focused on the histological and ultrastructural aspects of the intestinal tissue used to prepare explants. Thus, the aim of this study was to characterize these aspects in jejunal explants from 24- and 42-day-old pigs.

MATERIAL E MÉTODOS

Four weaned pigs, aged 24 (7.1 kg ± 0.9) and 42 (18.3 kg ± 1.7) days, were used in this study. The piglets were euthanized with an intravenous injection of sodium pentobarbital (40 mg/kg of body weight). The institutional Ethics Committee for Animal Experimentation approved all animal procedures.

Jejunum fragments (5 cm each) were collected in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 0.2 mL/L glutamine, 0.5 µg/mL gentamicin and 10 mL/L penicillin/streptomycin. Pieces with diameters of 8 mm were obtained with biopsy punches. Three explants/well were deposited villi upward onto agar-coated 6-well plates containing culture medium. The explants were incubated at 37°C with orbital shaking for 2 or 4 hours and subsequently fixed in 10% buffered formalin for histological analysis (hematoxylin-eosin staining). In all experiments, uncultured control tissue was placed into fixative solution at the end of the dissection time (0 h).

Histological changes were recorded, and a tissue score representing a maximum of 22 points was established based on the occurrence and severity of changes, as previously described (Kolf-Clauw *et al.,* 2009). Other criteria are added as enterocyte degeneration, microvilli preservation and

dilation of lymphatic vessels. Villi heights of ten randomly selected villi were measured using an image analysis system.

Explants from 24-day-old piglets were also subjected to ultrastructural analysis. The samples were fixed in Karnovsky modified solution, post-fixed in 1% osmium tetroxide, and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and analyzed by TEM (model FEI Tecnai 12).

The results are presented as the medians of independent variables. The scores and villi heights were analyzed by the Kruskal-Wallis test, followed by Dunn's test, using the BioEstat software 5.0. $P<0.05$ were considered significant.

RESULTADOS

We first evaluated the histological changes that occurred in the two age groups after two or four hours of incubation. The changes were compared to explants collected soon after death (control). Control explants from 24 and 42 day-old animals showed simple columnar cells with brush borders and interspersed goblet cells lining the gut (Figure 1A; D).

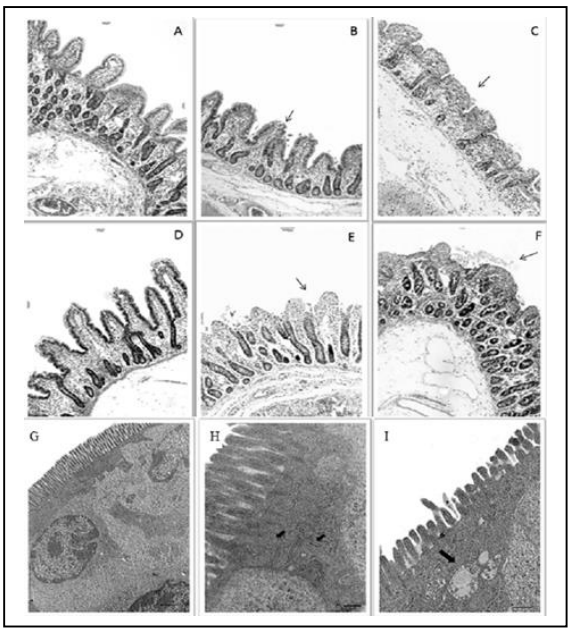

Figure 1 - Jejunal explants from piglets with 24 and 42 days old. A to C – 24 days old pigs and D to F – 42 days old pigs. (A) and (D) Non-incubated explants showing normal villi; (B) Explant after 2 hours of incubation. Mild villi atrophy (arrow); (C) Explant after 4 hours of incubation. Mild villi atrophy and fusion (arrow); (E) Explant after 2 hours of incubation. Mild villi atrophy and focal loss of apical enterocytes (arrow); (F) Explant after 4 hours of incubation. Severe atrophy and villi fusion, loss of apical enterocytes, and cellular debris (arrow) (H.E. Magnification 10x). G to I - Electron micrographs of explants after 4 hours of incubation. (G) Enterocyte monolayer lining the gut (Bar 2 µm). (H) Microvilli in the apical membrane of enterocytes (Bar 500 nm) (I) Endocytic vesicle in the apical cytoplasm (Bar 500 nm).

The intestinal villi showed normal morphology. A mild to moderate lymphocytic infiltration was observed in the lamina propria.

In incubated explants from 24-day-old piglets, the main changes were vacuolar degeneration of enterocytes and villi atrophy (Figure 1B; C). These changes increased from mild (2 h) to moderate severity (4 h). After four hours of incubation, mild villi fusion was also observed (Figure 1C). A significant decrease ($P<0.05$) in the tissue score of the explants from the 24-day-old group was verified at both time points (19% reduction after 2 h and 33.3% reduction after 4 h) compared to the control group (Figure 2A).

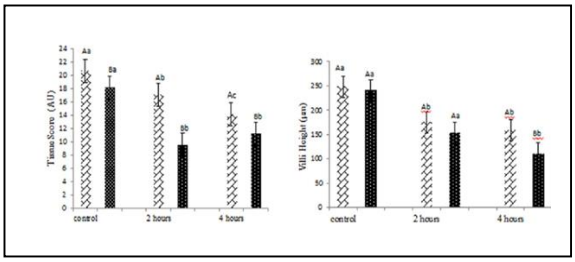

Figure 2 - Tissue score in jejunal explants from piglets with 24 and 42 days old in different times of incubation (A). Median villi height (µm) in jejunal explants from piglets with 24 and 42 days old (B). ▒ Piglets with 24 days old. ▉ Piglets with 42 days old. Different capital letters mean significant difference between ages. Different lower cases mean significant difference between control and incubated explants. ($p \leq 0.05$, Kruskall-Wallis Test). AU - arbitrary units.

After two hours of incubation, explants from the 42-day-old pigs displayed villi fusion and atrophy, with mild flattening and focal loss of apical enterocytes (Figure 1E). Similar changes were observed after four hours of incubation. In these explants, the tissue score decreased by 44.4% ($p \leq 0.05$) after two hours and by 38.8% ($P<0.05$) after four hours of incubation, in comparison with the control group (Figure 2A).

Villi height morphometry was performed to evaluate the maintenance of tissue morphology. The median villi heights in the control explants from 24- and 42-day-old pigs were 248.4 µm and 240.24 µm, respectively. Compared to control samples, a significant decrease in villi height was observed after two (29.6%) and four (35.7%) hours of incubation in the explants from 24-day-old piglets. The explants from 42-day-old pigs showed a significant reduction (53.9%) only after four hours of incubation (Figure 2B).

The histological scores and morphometrical measures were also compared between the age groups analyzed (24 and 42 days). The explants from 42-day-old pigs showed significantly lower jejunal scores than the explants from 24-day-old piglets at all-time points (0, 2 and 4 hours) (Figure 2A). The villi height was similar in the explants from both age groups at 0 and 2 hours of incubation. However, after 4 hours of incubation, the villi height from the 42-day-old pigs was significantly reduced (30.6%) (Figure 2B).

Ultrastructural analysis was performed to evaluate the tissue and cell integrity after incubation. We decided to use the samples with better histological preservation (from 24-day-old donors) from the 4-hour incubation group. The morphologies of the enterocytes, the cell membrane and the nuclear membrane were preserved. A continuous monolayer of enterocytes was observed lining the gut (Figure 1G), and the apical membrane formed numerous short microvilli (Figure 1H). Intercellular junctions and desmosomes in the apical region appeared between adjacent intestinal cells. The cell cytoplasm was rich in free ribosomes and glycogen granules. Endocytic vesicles were also observed in the apical cytoplasm (Figure 1I). The nuclei contained a low concentration of heterochromatin and large, distinct nucleoli. Goblet cells containing numerous secretory granules in the apical region were also observed.

DISCUSSION

Efforts to reduce the number of experimental animals have led to the development of new models laboratories. The explant model provides a system for evaluating numerous biological questions; however, it is limited by the short duration of tissue viability. Explants from the ureter, trachea and arteries can be maintained for periods of up to nine days (Trowell, 1959), but the viability of intestinal explants is relatively limited (Bansal *et al.,* 2009; Kolf-Clauw *et al.,* 2009). In this study, we observed that the age of the donor influenced the tissue viability, as histological score decrease was more pronounced in older pigs. Similar results have been reported in studies comparing tissues from embryos, weaning and young animals with those of older animals (Trowell, 1959; Kolf-Clauw *et al.,* 2009). Some studies have shown that cell regeneration and tissue integrity

under normal and hypoxic conditions are better in young animals than in older ones (Günther and kranz, 1981), leading to the hypothesis that increasing age is accompanied by a decreased ability to adapt to conditions such as hypoxia (Trowell, 1959; Stupina *et al.,* 1989).

We were also interested in evaluating the maximal incubation duration that preserves intestinal morphology and function. The intestinal morphology of the explants from 24-day-old piglets was preserved following incubation for two and four hours. On the other hand, after 4 hours of incubation, the histological score was 21.5% lower in samples from 42-day-old piglets than in samples from 24-day-old piglets. Villi atrophy and occasional villi fusion were the main microscopic findings after incubation. These findings are related to the hypoxic conditions that the explants experienced during the experiment. Time-dependent histological changes have also been reported in explants incubated for 4 to 8 hours (Kolf-Clauw *et al.,* 2009).

Ultrastructural analysis was performed to evaluate the tissue viability and function in incubated explants from 24-day-old piglets. As normal metabolism is necessary for morphological preservation, structural integrity reflects tissue homeostasis. Cell viability after 4 hours of incubation was confirmed by the presence of preserved cytoplasmatic and nuclear membranes and subcellular structures. The presence of adsorptive endocytosis in enterocytes indicates the maintenance of the intestine absorptive function. Mucin granules in goblet cells cytoplasm show the functionality of these cells, contributing to preservation of intestinal physical barrier.

Intestinal explants are more feasible than inoculations in animals, which are expensive to maintain, are time consuming to study, and require specific facilities. In this study, it was possible to obtain morphological and functional viability of the intestine when the tissue was obtained from 24-day-old animals and an incubation period of 4 hours was used. Our results demonstrated that the age of the donor is related to the resistance of the tissue to hypoxia. Other factors, such as additives in the culture medium, the temperature, and oxygen levels, can increase tissue viability and therefore improve the utility of explants in toxicological, nutritional or pathological studies.

CONCLUSÃO

It is concluded that physical exercise intensity cause different hemato-biochemical alterations in jump horses. Erythrocytes, PCV, ALP, creatinine and heart rate increase progressively with the exercise intensity. Hemoglobin and lactate increase only in jump competition. Total protein, CK and potassium increase in mounting exercise and jump competition. Glucose decreases in all the exercise protocols, while no alterations are observed in AST, urea and sodium.

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