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Vascular changes and acute inflammation induced by agar in the air pouch model

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ABSTRACT

Objective: To examine agar as a phlogistic agent in the air pouch model.
Method and Materials: Rats were divided into five groups and the effects of 1%, 2%, 3% and 4% agar in the air pouch model were compared with those of 1% carrageenan. The vascular inflammation induced by agar and the microvasculature of the air pouch membrane were analysed. In addition, the role of nitric oxide (NO)-dependent pathways and cellular migration in the responses to 2% agar were evaluated. To assess the mechanism of action underlying the inflammatory effects of agar, rats were treated with a standard anti-inflammatory drug, either celecoxib or acetylsalicylic acid. In addition, a differential leucocyte cell count, total cell count and NO and PGE_2 concentrations were determined.
Results: The 2% agar was chosen as the optimal concentration. Celecoxib or ASA both inhibited the inflammatory effects of agar, reducing the area of the microvasculature in the tissue lining the air pouch, as well as NO and PGE_2 concentrations and cell migration in the exudate.
Conclusion: The present study shows that a simple and alternative method (agar) produces consistent results in the air pouch model and can be used as an alternative experimental model of inflammation.

INTRODUCTION

Inflammation is the response of an organism to an injurious stimulus that initiates healing (Kenneth and Hajime, 2008). There are many mechanisms involved in inflammatory responses to infections, chronic diseases and other tissue injuries. This variety and the search for new anti-inflammatory drugs with higher specificity and fewer side-effects justify the development of new protocols and standardization of experimental inflammatory models (Kenneth and Hajime, 2008). The air pouch model was developed as an *in vivo* bioassay in rodents modelling a typical inflamed system (Bastos *et al.*, 2008; Tao *et al.*, 1999). The air pouch model of inflammation is widely used because it produces localized effects and is easy to create. The model enables easy collection of any inflammatory cells produced, as well as measurement of chemotaxis and the production of inflammatory chemical mediators (Bastos *et al.*, 2008; Tao *et al.*, 1999).

In a previous study, we demonstrated the effectiveness of an air pouch model with carrageenan as a bioassay to identify new agents with anti-inflammatory potential (Bastos *et al.*, 2008). In the present study, carrageenan, a phlogistic agent widely used in different experimental models, was replaced by agar in the air pouch model in rats on the basis that agar is a hydrocolloid extracted from red algae (*Phylum Rhodophyta*), the same phylum from which carrageenan is obtained.

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Agar is a substance that is widely used in the food, pharmaceutical and cosmetic industries; like gums and pectins, agar is a vegetable gum. Previously, Okoli *et al*. evaluated the oedamatogenic action of agar in the paw oedema induction model (Okoli et al., 2006). The effect of non-steroidal anti-inflammation produced in the air pouch is a very reproducible way of study in the cellular and vascular components of inflammation (Sedgwick *et al.*, 1984). In general, effects of the standard non-steroidal anti-inflammatory drugs (NSAIDs) celecoxib (a selective cyclo-oxygenase (COX)-2 inhibitor) and acetylsalicylic acid (ASA; a non-selective COX-1/COX-2 inhibitor) are used to investigate the mechanisms involved in models of inflammation (Meng *et al.*, 2015).

Thus, the aim of the present study was to evaluate the use of agar as a phlogistic agent in the air pouch model to enable studies of nitrergic activity, PGE_2 concentrations, cell migration, total and differential leucocyte cell counts and morphometric analysis of the area of extent of the microvasculature in the tissue lining the air pouch.

METHODS

Materials

The following reagents were used in the present study (all from Sigma Chemical, St Louis, MO, USA): agar (A5306), k-carrageenan (22048), celecoxib (PZ0008), ASA (A5376), phosphoric acid (V000145), sulphanilamide (33626), *N*-(1-naphthyl) and ethylenediamine dihydrochloride (222488).

Animals

Male Wistar rats (150–200g) were purchased from the Animal Center of the Federal University of Pará (Belém, Brazil) and maintained in experimental animal facility from the Institute of Biological Sciences (n = 36). Rats were housed in a temperature-controlled room (22±1°C) under a 12 h light–dark cycle, with water available *ad libitum*. The present study was conducted in accordance with the guidelines of the Ethics Committee for Research with Experimental Animals of the Federal University of Pará (CEPAE-UFPA; 111-13). All efforts were made to minimize the number of animals used and their suffering.

Preparation of air pouches and induction of inflammation

Air pouches were created as described by Tao *et al.* (1999) and Bastos *et al.* (2008). Briefly, air was carefully removed from inside a flow chamber to avoid contamination. The pouches were inflated with 20 mL air injected into the intrascapular area and then re-inflated with 10 mL air every 3 days.

Three days after the creation of the air pouches, inflammation was induced by subcutaneous route (s.c.) of different concentrations of agar (1-4%) or 1% carrageenan-kappa in 0.9% NaCl (saline) inside the cavity, except in the control group, which received an injection of 0.25 mL of saline.

Sample collection

Samples of exudate were obtained 16 h after administration of agar or carrageenan. To this end, a small incision was made in the wall of the air pouch and the contents were carefully removed using a sterile Pasteur pipette. To increase the total volume of the exudate and thus improve the precision of measurements, 3 mL phosphate-buffered saline (PBS; composition (in mmol/L): PO_4^{3-} 10; NaCl 137; KCl 2.7, pH 7,4) had been injected into the air pouch. After collection of the exudate, nitrite content, the total number of cells, the leucocyte cell count and PGE_2 concentrations were determined in the samples.

Morphometric analysis of extent of the microvasculature

Screening and automatic quantification of morphological features were used to determine the efficacy of pharmacological perturbation of inflammatory processes. The features evaluated were the extent of the microvasculature.

The mean area of extent of the microvasculature was measured using NeuronJ (Departments of Medical Informatics and Radiology Rotterdam, The Netherlands) (Meijering *et al.*, 2004) a semi-automated nerve-tracing software package, a plug-in module for ImageJ (National Institutes of Health, Bethesda, MD, USA), a free Java-based image analysis software package (Meijering, 2010). Extent of the microvasculature was quantified following identification of the microvasculature in the area of the air pouch. Briefly, after removal of the exudate, the tissue lining the dorsal area of the air pouch was examined to evaluate microvasculature. The microvasculature was photographed using a digital camera (DSC-H200; Sony, Tokyo, Japan). The camera was supported on a mini tripod at a constant distance of 20 cm above the rat. Images were recorded at a resolution of 345 × 440 pixels, covering an area of 625 × 525 mm.

Labelled microvasculature was traced manually, and an algorithm was used to compare the pixel intensity on the microvasculature with pixels in adjacent areas, automatically updating the cursor to follow the predicted path, increasing the accuracy and speed of tracing. After the microvasculature had been traced, a text file was generated containing the lengths of all the microvasculature by NeuronJ.

The microvasculature tracings appeared coloured (pink). After marking of the microvasculature, length measurement data were generated from the microvasculature and an image was stored and analysed in uncompressed 8-bit Tiff format. The program performed arithmetic calculations on the measurement data generated by NeuronJ and wrote the calculations directly into an Excel (Microsoft, Bellevue, WA, USA) worksheet, with mean area of each microvasculature which was measured in square millimeters (mm^2).

Nitrergic activity

Exudate samples were diluted in PBS in a ratio of 1:2. Subsequently, 500 µL aliquot of each diluted sample was mixed with the same volume of Griess reagent (0.1% naphtylethylene,

1% sulphanilamide in 5% phosphoric acid) (Green et al,. 1982). Optical density was determined at a wavelength of 570 nm to determine NO_2^-/NO_3^- concentrations using a standard curve (0, 2, 5, 10, 25, 50 µl) constructed using sodium nitrite solution as the standard.

Effects of standard anti-inflammatory drugs on agar-induced inflammation

To evaluate the role of COX-1 and COX-2 in the inflammation induced by 2% agar, 200 mg/kg celecoxib (selective COX-2 inhibitor) or 100 mg/kg ASA (non-selective COX inhibitor) in 0.9% saline were administered to rats orally 1 h before the injection of 2% agar.

Inflammatory exudate cell analysis

The inflammatory exudate was stained by MayGrünwald-Giemsa method to differential cell counts. The differentiated cells were determined by counting of 400 cells in which field under light microscope. The cells were identified by standard morphological criteria, nuclear morphology, and cytoplasmic granulation. Cell counts were expressed as percentages of total cells and absolute values (10^6/mL).

Determination of PGE_2 concentrations

Exudate samples were diluted 1:10 in PBS. PGE_2 concentrations in the samples were quantified using a specific PGE_2 ELISA (GE Healthcare UK, Little Chalfont, UK) according to Protocol 1 of the manufacturer's instructions. Optical density was determined at a wavelength of 570 nm.

Statistical analysis

All data are presented as the mean ± SD. The significance of differences between groups was examined using repeated-measures analysis of variance (ANOVA) and Tukey's multiple comparison test correction. Analyses were performed in GraphPad Prism version 5.00 (GraphPad Software, San Diego California USA). Two-sided $P < 0.05$ was considered significant.

RESULTS

Tissue microvasculature

The effects of agar on the microvasculature in the air pouch model were evaluated using the NeuronJ (Departments of Medical Informatics and Radiology Rotterdam, The Netherlands) semi-automated nerve-tracing software package (Meijering *et al.*, 2004), a plug-in module for ImageJ (National Institutes of Health, Bethesda, MD, USA) (Meijering, 2010). As shown in Fig. 1a and 1b, following creation of the air pouch, the control group (0.9% saline) exhibited normal microvasculature development. A macroscopic view of the air pouch after injection of 1% carrageenan into the intra-scapular area of rats is shown in Fig. 1c and 1d; in this group, carrageenan induced increased microvasculature development compared with the control group, injected with 0.9% saline. The effects of injection of 1%, 2%, 3% and 4% agar on the microvasculature are shown in Fig. 1e–ll. The effect of agar on the development of the microvasculature was observed. Interestingly, 4% agar increased the number of ischaemic points and a foul odour was noted after opening the air pouch in this treatment group.

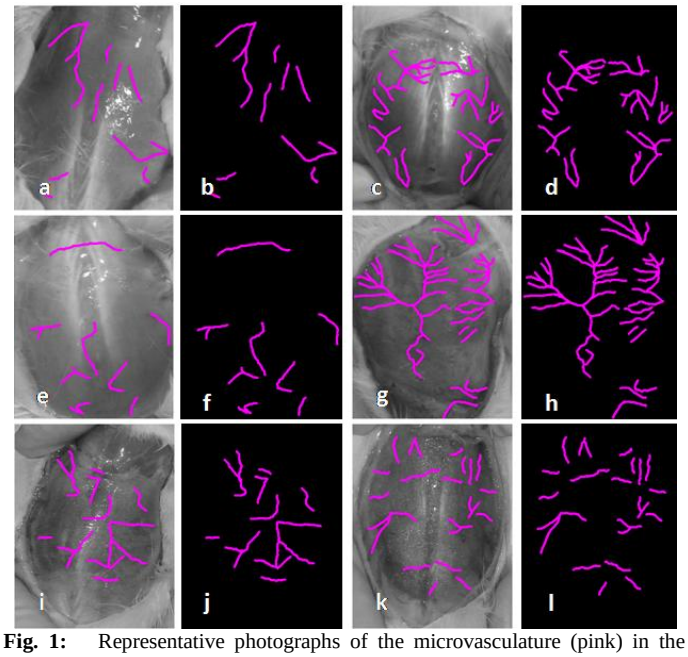

Fig. 1: Representative photographs of the microvasculature (pink) in the tissue lining the inside skin of the air pouch. The Fig. 1 (a, c, e, g, i and k) show the microvasculature used to obtain the means of extent of the microvasculature, while the Fig. 1 (b, d, f, h, j and l) show color images (pink) from tracings through NeuronJ. In the (a) and (b) control (0.9% saline), (c) and (d) 1% carrageenan, (e) and (f) 1%, (g) and (h) 2%, (i) and (j) 3%, (k) and (l) 4% agar groups. The tracings were adapted from images obtained using the NeuronJ (Departments of Medical Informatics and Radiology Rotterdam, The Netherlands) software package. These features include the length of the extent of the microvasculature.

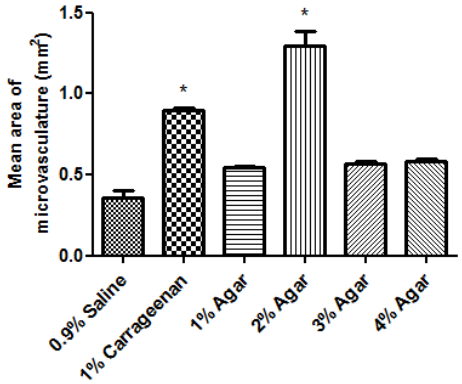

Fig. 2: Quantification of the mean area of extent of the microvasculature in the lining of the air pouch based on images obtained using the NeuronJ (Departments of Medical Informatics and Radiology Rotterdam, The Netherlands) software package in the control (0.9% saline), 1% carrageenan and 1%, 2%, 3% and 4% agar groups. Data are the mean ± SEM of four rats per group. *$P < 0.05$ compared with the control group (repeated-measures ANOVA and Tukey's multiple comparison test correction).

The mean area of the microvasculature based on the images obtained in the different groups is shown in Fig. 2. The area of the microvasculature increased almost twofold in the 1% carrageenan

compared with control group (0.889 *vs* 0.314 mm^2, respectively). In the 2% agar group, there was an almost threefold increase in the area of the microvasculature (1.211 mm^2) compared with the control group. Interestingly, injection of 3% and 4% agar resulted in only modest increases in the inflamed area (0.547 and 0.563 mm^2, respectively), which did not differ significantly compared with the control group. Morphometric analysis by identification of the extent of the microvasculature in the air pouch was used to evaluate the vascular effects of standard NSAIDs on the inflammation induced by agar. Figure 3 shows representative images of the process of extent of the microvasculature induced by 0.9% saline (Fig. 3a and 3b) and 2% agar alone (Fig. 3c and 3d) and after pretreatment with 200 mg/kg celecoxib (Fig. 3e and 3f) or 100 mg/kg ASA (Fig 3g and 3h).

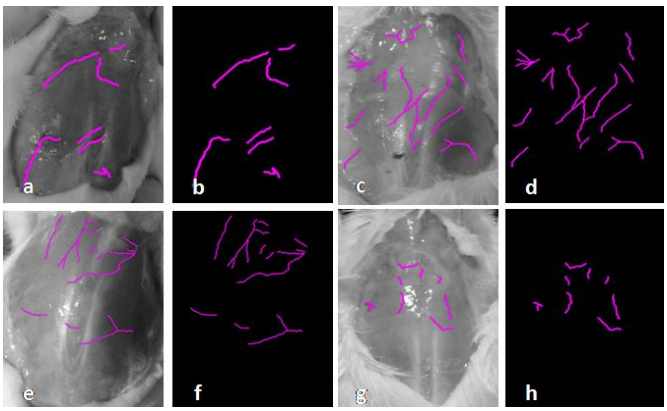

Fig. 3: Representative photographs of the microvasculature (pink) in the tissue lining the air pouch. The Fig. 3 (a, c, e and g) show the microvasculature used to obtain the means of extent of the microvasculature, while the Fig. 3 (b, d, f and h) show color images (pink) from tracings through NeuronJ. In the (a) and (b) control (0.9% saline), (c) and (d) 2% agar group, (e) and (f) 200 mg/kg celecoxib-pretreated agar group and (g) and (h) 100 mg/kg acetylsalicylic acid (ASA)-pretreated agar group. The tracings were adapted from images obtained using the NeuronJ (Departments of Medical Informatics and Radiology Rotterdam, The Netherlands) software package. These features include the length of the extent of the microvasculature.

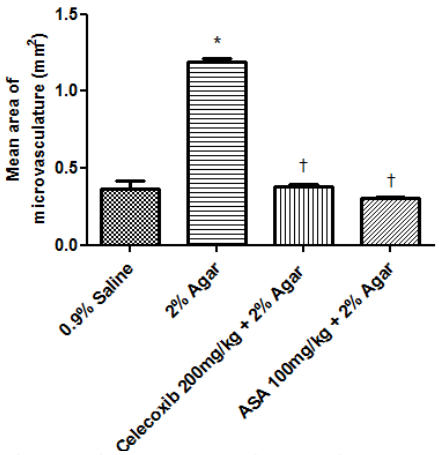

Fig. 4: Quantification of the mean area of extent of the microvasculature in the lining of the air pouch based on images obtained using the NeuronJ software package in the control and 2% agar groups and the effects of 200 mg/kg celecoxib and 100 mg/kg acetylsalicylic acid (ASA) on the responses to 2% agar. Data are the mean ± SEM of four rats per group. **P* < 0.05 compared with the control group; †*P* < 0.05 compared with the 2% agar group (repeated-measures ANOVA and Tukey's multiple comparison test correction).

A summary of the mean area of the microvasculature calculated from these images is shown in Fig. 4. Pretreatment with celecoxib or ASA reduced the area by approximately one-third compared with 2% agar alone (0.393 or 0.300 *vs* 1.176 mm^2, respectively; *P* < 0.05). There was no significant difference in the mean area of the microvasculature between the celecoxib- and ASA-pretreated groups.

Role of NO-dependent pathway in agar-induced inflammation

To determine whether the NO pathway plays a role in the inflammatory response induced by agar, the production of NO_2^- /NO_3^- in inflammatory exudates was evaluated. As shown in Fig.5a, agar (1%, 2%, 3% and 4%) significantly increased NO_2^- /NO_3^- concentrations compared with control (13.7 ± 1.8, 22.9 ± 1.5, 32.8 ± 1.8 and 26.9 ± 0.5 vs 1.78 ± 1.05 µmol/L, respectively; P < 0.05 for all). Injection of 1% carrageenan also increased NO_2^- /NO_3^- concentrations in the exudate (25.8 ± 3.6 µmol/L; P < 0.05 compared with control). There were no significant differences among the 2% and 4% agar and 1% carrageenan groups.

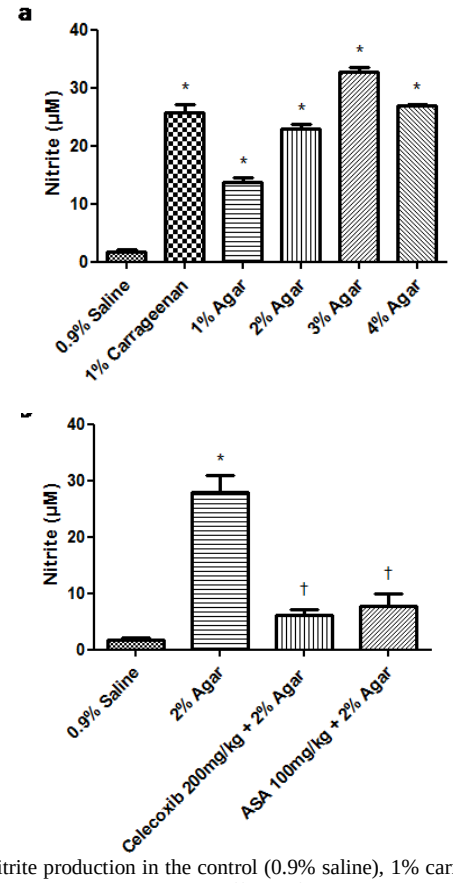

Fig. 5: (a) Nitrite production in the control (0.9% saline), 1% carrageenan and 1%, 2%, 3% and 4% agar groups. (b) Effects of 200 mg/kg celecoxib and 100 mg/kg acetylsalicylic acid (ASA) on nitrite production in response to 2% agar. Data are the mean ± SEM of four rats per group. **P* < 0.05 compared with the control group; †*P* < 0.05 compared with the 2% agar group (repeated-measures ANOVA and Tukey's multiple comparison test correction).

To evaluate the effects of the standard NSAIDs against the inflammation induced by 2% agar, NO_2^-/NO_3^- concentrations were determined in rats pretreated with 200 mg/kg celecoxib or

100 mg/kg ASA. As shown in Fig. 5b, celecoxib or ASA decreased NO_2^-/NO_3^- concentrations by 85% and 81%, respectively, compared with 2% agar alone. The corresponding concentrations of NO_2^-/NO_3^- in these three groups were 6.1 ± 2.2 and 7.7 ± 5.1 versus 27.9 ± 6.9 µmol/L, respectively.

Cellular migration in the air pouch model after agar injection

To determine whether cells migrated from the circulation to the injury site, we evaluated the total number of cells present in the exudate after agar injection. As shown in Fig. 6a, agar (1%, 2%, 3% and 4%) significantly increased the total number of cells in the exudate compared with control (7 ± 2, 9 ± 3, 15 ± 2 and 13 ± 3 *vs* 1.1 ± 0.3 $\times 10^7$ cells/mL, respectively; $P < 0.05$ for all). Injection of 1% carrageenan also increased the total number of cells in the exudate (8.1 ± 0.9 $\times 10^7$ cells/mL; $P < 0.05$ compared with control).

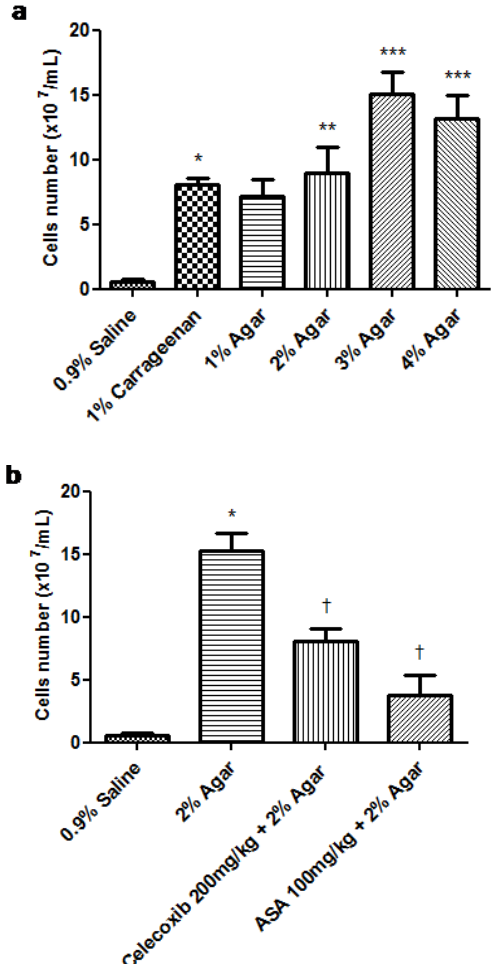

Fig. 6: (a) Cellular migration in the control (0.9% saline), 1% carrageenan and 1%, 2%, 3% and 4% agar groups. (b) Effects of 200 mg/kg celecoxib and 100 mg/kg acetylsalicylic acid (ASA) on cellular migration in response to 2% agar. Data are the mean ± SEM of four rats per group. *$P < 0.05$, **$P = 0.0032$, ***$P < 0.001$ compared with the control group; †$P < 0.05$ compared with the 2% agar group (repeated-measures ANOVA and Tukey's multiple comparison test correction).

Pre-treatment of rats with 200 mg/kg celecoxib or 100 mg/kg ASA prior to 2% agar injection decreased the total number of cells in the exudate by 47% and 73%, respectively, compared with agar alone. The corresponding number of cells in the exudate in these three groups was 8.0 ± 2.2 and 3.7 ± 2.8 *vs* 15.2 ± 3.0 $\times 10^7$ cells, respectively. The difference in the inhibitory effects of celecoxib or ASA did not reach statistical significance.

Differential leucocyte cell count in the inflammatory exudates

A differential leucocyte count was performed to identify the cell types present in the exudate after injection of 2% agar (Fig. 7). As indicated in Fig. 8, the exudate contained a large population of neutrophils, followed by monocytes/macrophages and a small amount of lymphocytes and eosinophils (63.3 ± 23.4, 27.3 ± 2.5, 9.3 ± 11.1 and 8.7 ± 10.1 $\times 10^6$ cells/mL, respectively).

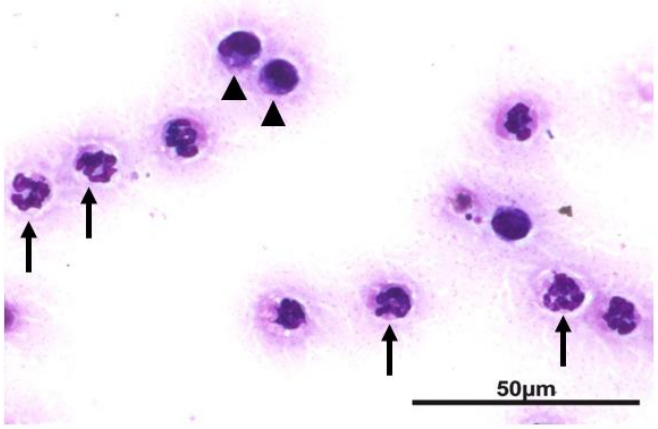

Fig. 7: Light microscopy of Giemsa stained exsudate from air pouch. Representative image of the differential leukocyte cell count present in the inflammatory exudates of 2% agar group. The arrowhead indicate monocytes/macrophages (showing kidney shaped nucleus), while the arrow indicate neutrophils (nucleus with three to five lobes). *Bar* 50 µm.

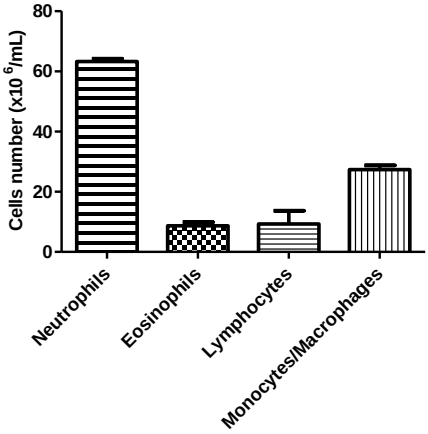

Fig. 8: Differential leukocyte cell count, expressed as the total number of cells per mL exudate, after injection of 2% agar into the air pouch. Data are the mean ± SEM of four rats per group.

Effects of NSAIDs on PGE_2 concentrations after 2% agar injection

Concentrations of PGE_2 were determined in the exudate of the control and 2% agar and 1% carrageenan-injected groups. As indicated in Fig. 9, injection of agar or carrageenan significantly increased PGE_2 concentrations compared with the

control group. Pretreatment of rats with 200 mg/kg celecoxib or 100 mg/kg ASA prior to injection of 2% agar significantly reduced PGE_2 concentrations in the exudate compared with agar alone (Fig. 9). There was no significant difference in the inhibitory effect of celecoxib and ASA.

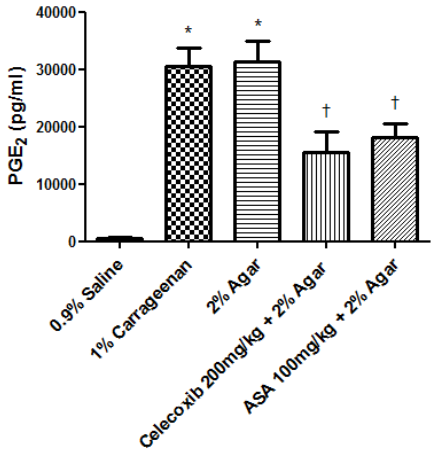

Fig. 9: PGE_2 concentrations in the control (0.9% saline), 1% carrageenan and 2% agar groups, and 2% agar groups pretreated with 200 mg/kg celecoxib or 100 mg/kg acetylsalicylic acid (ASA). Data are the mean ± SEM of four rats per group. $^*P < 0.05$ compared with the control group; $^\dagger P < 0.05$ compared with the 2% agar group (repeated-measures ANOVA and Tukey's multiple comparison test correction).

DISCUSSION

Inflammation is a part of complex biological response of vascular tissues to harmful stimuli, such as pathogens or irritants. Inflammation models are very important for the discovery of new drugs. In the present study we characterized the phlogistic effect of agar as an alternative agent in the air pouch model. To that end, we evaluated the effects of agar injection on nitrergic activity, cellular migration, the leucocyte count, PGE_2 concentrations and vascular changes (microvasculature in the tissue lining the air pouch).

Using different procedures to create experimental models of inflammation has helped elucidate the mechanisms underlying the swelling, heat, pain, redness and loss of function associated with inflammation (Parnham, 2008). The air pouch model with carrageenan is one of the most commonly used models because it allows for a broader analysis of inflammatory signals (Jain and Parmar, 2011; Leclerc *et al.*, 2013). Our use of agar as an alternative in the air pouch model is supported by the findings of Okoli *et al*. (2007), who used agar to create paw oedema in rats. The present study is the first to characterize the responses of different inflammatory parameters to agar injection in the air pouch model.

On the basis of the initial experiments evaluating 1%, 2%, 3% and 4% agar, 2% agar was chosen as the optimal concentration because there was an almost threefold increase in the area of the microvasculature, the values of NO and cell migration were better when compared than the 1% and 3% agar groups. These amounts were not better than those found in 4% agar group, but in this concentration, there was an increased the number of ischaemic points and resulted in a foul odour when the air pouch was opened. The presence of ischaemia and the foul odour are evidence of loss of function, a negative sign in models of inflammation, because dying cells are capable of activating the innate immune system and inducing an intense inflammatory response (Freire and Van Dyke, 2013).

Furthermore, agar increased the area of extent of the microvasculature inside the air pouch, as well as nitrite concentrations. It is important to emphasise that NO increases vascular permeability because it is a potent vasodilator (Davis *et al.*, 2001). In the present study, injection of agar into the air pouch cavity increased nitrite concentrations. These results are in agreement with those of Szabó and Horton (2003) who demonstrated that during the first hours after an injury capable of initiating an inflammatory process, NO production mediated by inducible NO synthase (iNOS) can be upregulated, resulting in the release of large amounts of NO, and that this upregulation of NO, in turn, causes cellular damage.

Cellular migration may be another important consequence of increased NO as part of the inflammatory process induced by agar. According to Szabó and Bechara (1999), during the acute inflammatory process, microvasculature diameter, blood flow and vascular permeability are modified, triggering cellular migration. Nitric oxide can directly increase peripheral extent of the microvasculature, resulting in vascular decompensation; this effect can be trigged by the nuclear factor (NF)-κB signalling pathway, leading to the production of proinflammatory cytokines (Szabó and Horton (2003). In addition, Salvemini *et al.* (2006) used iNOS inhibitors in the air pouch model and observed that the inhibitors had an anti-inflammatory action, not only because iNOS was blocked, but also because of a decrease in the cellular infiltrate. Therefore, nitrite levels and vascular effects are likely to be closely related, and this is in agreement with the findings reported herein.

Determination of the differentiated leucocyte cell count in the 2% agar group revealed a prevalence of neutrophils, followed by monocytes/macrophages and small numbers of lymphocytes and eosinophils. Garcia-Ramalho *et al.* (2002) demonstrated that, during inflammation, resident cells induced the release of tumour necrosis factor (TNF)-α, which is responsible for chemokine synthesis and subsequently leucocyte migration. Other cytokines, such as interleukin (IL)-1 and IL-6, also participate in this process (Michaud *et al.*, 2013). During the development of inflammation, leucocyte migration is initiated by a significant number of polymorphonuclear (PMNs) neutrophils followed by monocyte/ macrophage migration (Cox *et al.*, 2013). The increased leucocyte migration may be due to induction of adhesion molecule expression, which is responsible for the 'rolling' of leucocytes along the endothelium (Martin *et al.*, 2000). This process can be explained by activation of nuclear transcription factors, such as NF-κB, which is a key protein responsible for the gene transcription of iNOS, COX-2 and

vascular cell adhesion molecule (VCAM)-1(Kim *et al.*, 2010). The inflammatory mediators bind to receptors present on macrophages, resulting in the phosphorylation and degradation of IκB and translocation of NF-κB to the nucleus, where it binds to DNA promoter regions, initiating the transcription of genes encoding other inflammatory mediators (Laskin and Laskin, 2001).

The effects of celecoxib or ASA were evaluated in the air pouch model induced by agar. Celecoxib, a selective COX-2 inhibitor, and ASA, an NSAID, inhibited the inflammation induced by agar. The anti-inflammatory action of ASA may involve inhibition of the catalytic activity of COX-1, which produces PGE_2 and thromboxanes (Liu *et al.*, 2012). However, ASA and other derivatives of standard NSAIDs do not inhibit lipo-oxygenase, and thus do not suppress the formation of leukotrienes (Köhnke *et al.*, 2013). Celecoxib exhibits anti-inflammatory, antipyretic and analgesic properties attributed to selective inhibition of COX-2 (Park and Lee, 2005; Price and Jorgensen, 2001). A previous study has demonstrated that NSAIDs significantly inhibit pain induction (Bastos *et al.*, 2006).

Chemical mediators, including histamine, NO, PGE_2 and prostacyclin (PGI_2), promote extent of the microvasculature. Furthermore, PGE_2 and PGI_2 cause redness and heat in the tissue by increasing local blood flow. In a qualitative test, greater extent of the microvasculature and redness were observed following injection of 2% agar compared with the control group, suggesting that agar injection induced a greater release of inflammatory mediators. However, PGE_2 concentrations were similar in the NSAIDs groups, indicating a similar mechanism of action. Pretreatment with celecoxib or ASA inhibited the extent of the microvasculature and redness in response to 2% agar. The ability of celecoxib to reduce the extent of the microvasculature and redness in response to injection of 2% agar suggests that agar may induce COX-2 in inflammation (Hilário *et al.*, 2006).

In the present study, both standard NSAIDs blocked the effects of 2% agar on nitrergic activity and PGE_2 concentrations. Jung *et al.* (2011) analysed the anti-inflammatory effects of *n*-propyl gallate through downregulation of NF-κB in a cultured RAW 264.7 cell line, and concluded that the decrease in nitrite concentrations after exposure of cells to *n*-propyl gallate could be an indicator of anti-inflammatory activity. The observations in the present study could be explained by inhibition of NF-κB. Time-dependent induction of NF-κB has been demonstrated in carrageenan-induced inflammation in the air pouch model and NF-κB expression has been shown to be inhibited using dexamethasone (a steroidal anti-inflammatory drug) (Crippen, 2006; Ellis *et al.*, 2000).

In another study, Meacock and Kitchen (1976) tested the effects of various NSAIDs, such as indomethacin, phenylbutazone, ketoprofen, ibuprofen, ASA, naproxen and fenoprofen, on cellular migration and concluded that none of the drugs tested prevented the migration of polymorphonuclear leucocytes in carrageenan-induced inflammation. However, the NSAIDs tested suppressed the migration of mononuclear cells (monocytes). The decreased leucocyte migration in the NSAIDs groups observed in the present study could be due to blockade of the expression of adhesion molecules, which are responsible for the 'rolling' of leucocyte along the endothelium. The blockade of the expression of these molecules can be explained by inhibition of transcription factors, such as NF-κB, which is a key factor responsible for the gene transcription of iNOS, COX-2 and VCAM-1 (Köhnke *et al.*, 2013; Coleman, 2001). In conclusion, the air pouch protocol described herein may provide a simple alternative for studies involving inflammation. It may also prove useful in the screening of new anti-inflammatory drugs, including determining potential targets of action.

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