Original article

RGB stacks gray-level analysis to study mast cell degranulation to intestinal contraction correlation in trout

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Abstract

Introduction: To date a reliable method to correlate degranulation-related depletion of histochemically evidenced components of mast cell cytoplasm to intestine contraction is lacking. Degranulation of intestinal mast cells in rainbow trout was studied *ex vivo* by means of image analysis technique and correlated to the maximal intestinal contraction elicited by degranulation itself. **Materials and Methods:** Two strips from the same intestinal segment from ten trout were sampled. One was immediately mounted in an organ bath, to test the effect of incremental doses of compound 48/80, then processed, as the remnant untreated strip, for light microscopy (Giemsa stained). Color pictures from each section were converted in their gray levels RGB stacks equivalent and in eight bits gray levels. Five granular cytoplasm areas of mast cells for each section were analyzed for mean gray values. **Results and Conclusion:** Differential mean gray values (after compound 48/80 - before compound 48/80) of Green (R^2 0.84, $p < 0.01$) and Blue (R^2 0.83, $p < 0.01$) channels and eight bits grayscale (R^2 0.76, $p < 0.05$) correlated significantly with the respective value of maximal intestinal contraction, "controlling for" the effect of the mean gray value before compound 48/80 addition. Basing upon the results, a possible acidic/anionic pro-contractile agonist can be inferred. The proposed *ex vivo* model and RGB stacks gray-level analysis represent a promising tool to study the role of piscine mast cell in intestine contractile modulation.

Keywords: densitometry, histochemistry, isolated organ bath, *ex vivo* model, Giemsa.

1 Introduction

Mast cells (**MCs**) of fish have been previously named eosinophilic granule cells (**EGCs**) because of the affinity of their granule to eosin, this fact causing misleading and contradicting interpretation (SIRE and VERNIER, 1995; REITE, 1997; REITE and EVENSEN, 2006). MCs of Salmoniformes form characteristically large clusters in the so named *stratum granulosum* of the gastrointestinal tract (ELLIS, 1977; EZEASOR and STOKOE, 1980; REITE and EVENSEN, 2006). Considering mast cell and its degranulation, rainbow trout (*Oncorhynchus mykiss*, Walbaum) has been considered a possible alternative to zebrafish (*Danio rerio*, Hamilton), because of the presence of a *stratum granulosum*, which makes identification of mast cells and the application of densitometry on them easier and because of the compliance with "The three R's" principles and European Community regulation on experimental animal welfare (MANERA and BORRECA, 2012).

Research on piscine MCs has principally focused on their involvement in immunity (REITE, 1998; REITE and EVENSEN, 2006; LEKNES, 2007) and antimicrobial peptides (piscidins), have been also detected in them, according to fish species (SILPHADUANG, COLORNI and NOGA, 2006). Only recently studies have appeared about their possible role in fish intestinal motility (MULERO, PILAR SEPULCRE, MESEGUER et al., 2007; MANERA, GIAMMARINO, BORRECA et al., 2011). In effect, the role of mammalian MCs in the modulation of intestinal motility is known (STENTON, VLIAGOFTIS and BEFUS, 1998; RIJNIERSE, NIJKAMP and KRANEVELD, 2007; VAN NASSAUW, ADRIAENSEN and TIMMERMANS, 2007).

In a previous research paper, maximal intestinal contraction has been shown to correlate with degranulation intensity, as a result of compound 48/80 administration, and assessed by means of mean gray values analysis (densitometric analysis) in an *ex vivo* experimental model in rainbow trout (MANERA, GIAMMARINO, BORRECA et al., 2011). In addition, densitometric analysis has been performed to discriminate degranulation status in an experimental ex vivo animal model with rainbow trout intestinal strips. The latter provided quali-quantitative data on mast cell degranulation and a promising alternative in mast cell research (MANERA and BORRECA, 2012). Moreover and in particular, **RGB** (Red Green Blue color representation) channels provided better discrimination power to distinguish degranulated and non-degranulated mast cells in trout intestine, compared to grayscale images, in consideration of the inevitable loss of information passing from the original RGB color images to grayscale (MANERA and BORRECA, 2012).

Scope of the present survey is to provide morpho-functional correlates (degranulation *versus* intestinal contraction) in rainbow trout *ex vivo* model by means of gray-level analysis according to RGB channels and to gain histochemical information about the putative pro-contractile

agonist, useful to plan further morpho-functional research and to get novel information about the role of mast cell in intestine contractile modulation. In particular, depletion of histochemically evidenced component of mast cell cytoplasm due to degranulation was correlated to the evoked intestine contraction in a cause-effect relationship. Gut functionality is the prerequisite for nutrients absorption and their transformation in trout edible body mass. Therefore, any information regarding trout intestine function is mandatory for an efficient trout farm activity.

Compound 48/80, a condensation product of N-methyl-p-methoxyphenethylamine with formaldehyde (PATON, 1951) was adopted as MCs degranulation agent.

2 Materials and Methods

2.1 Fish selection

Ten clinically healthy *O. mykiss* were sampled during regular slaughtering. Fish were fasted 24 h prior to sampling and were stunned in ice plus water, followed by spinalization and brain disruption. A necropsy was performed on each fish, to exclude gross pathology with particular attention to the intestine. Moreover, and in order to assess fish health, blood samples were collected from all the examined fish, by means of caudal vein puncture. Serum parameters appeared to be in the range of normality according to Manera and Britti (2006, 2008).

2.2 Ex vivo experiment

Details on the adopted *ex vivo* technique, with particular regard to dose-response curve, have been previously reported (MANERA, GIAMMARINO, BORRECA et al., 2011) and are summarised below.

For each fish, two strips were sampled from the same segment of the mid-intestine. One strip of the two was immediately mounted in an organ bath containing 10 ml trout Ringer's solution, aerated with a mixture of O_2 and CO_2. The remnant strip was immediately fixed in 10% neutral buffered formalin solution. At the end of each trial, intestinal strips were removed from the organ baths, and immediately fixed in 10% neutral buffered formalin solution. Intestinal strips mounted in the organ baths were allowed to equilibrate for one h, and rinsed with fresh trout Ringer solution every 20 min. The agonist, compound 48/80 (Sigma-Aldrich Co., Milan, Italy) was added to the bath with a defined concentration progression according to a cumulative dose-response study.

Reader is referred to previously mentioned paper (MANERA, GIAMMARINO, BORRECA et al., 2011) for any further details on the adopted *ex vivo* technique.

2.3 Histology and gray values analysis

Formalin-fixed strips were processed for light microscopy by dehydration through an ethanol series and embedded in paraffin. Sections (5 μm) were deparaffinized with xylene and hydrated to distilled water through an ethanol series; then they were stained in 20% Giemsa solution (eosin methylene-blue solution according to Giemsa; Merck KGaA, Darmstadt, Germany) for 75 min; rinsed in distilled water (three changes) to remove the excess of stain; rinsed in acetic acid solution (three drops of glacial acetic acid in 250 mL of distilled water) for 150 sec; differentiated in 95% and absolute ethanol for one min; cleared with xylene and mounted.

Sections were observed and photographed (100 x, oil-immersion) with a digital colour camera (DS-5M; Nikon, Tokyo, Japan) manually set to ensure the same exposure parameters, light intensity and white balancing. Images were saved as TIFF (RGB – Red, Green, Blue method) lossless format and analyzed with a public domain Java image processing program (ImageJ 1.46d; Rasband W., National Institute of Health, USA). For each of 20 sections (10 before and 10 after compound 48/80 addition), five granular cytoplasm areas of MCs were randomly selected by means of the freehand selection instrument of ImageJ. Thereafter, the original colour pictures were converted in their gray levels RGB stacks equivalent. Namely the Red, Green, Blue channels were singularly converted in eight bits gray levels (grayscale conversion). Original 24 bit colour RGB pictures were also converted in eight bits gray levels using the following standard formula: eight bit gray = (eight bit Red channel + eight bit Green channel + eight bit Blue channel)/3 (FERREIRA and RASBAND, 2011). Mean gray values were recorded from the selected area of each channel (Red, Green, Blue grayscale converted channels and original colour RGB grayscale converted) for each treatment group (before and after compound 48/80 addition).

2.4 Statistical analysis

Differential mean gray value (Δ), namely the difference of the mean gray value after compound 48/80 addition with the respective mean gray value before compound 48/80 addition, from each of the tested intestinal strip and for each of the grayscale RGB channel, were correlated by means of partial correlation method with the respective values of maximal intestinal contraction recorded during the *ex vivo* experiment, in order to gain information about the histochemical nature of the involved pro-contractile agonist.

In particular, differential mean gray value of each strip was correlated with the respective value of maximal intestinal contraction, "controlling for" the effect of mean gray value before compound 48/80 addition, to avoid the occurrence of spurious correlation. Assumption of data normality was previously assessed by means of Kolmogorov-Smirnov and Shapiro-Wilk test. Data were normally distributed. SPSS® 14.0.2 (SPSS Inc., Chicago, IL, Usa) was used as the statistical package for means comparison and correlation.

3 Results

Giemsa staining technique enhanced blue coloration of mast cell granules in trout, therefore Red and Green channels ensured the best visual contrast at light microscopy, particularly in MCs from strips of untreated intestine with the following order of contrast intensity: Red channel converted in eight bit gray levels, Green channel converted in eight bit gray levels, the original RGB colour image converted in eight bit gray levels (grayscale) and Blue channel converted in eight bit gray levels. In Figure 1 clusters of MCs are reported according to treatment (before and after compound 48/80 addition) and to RGB channels. Gray values of five randomly selected granular cytoplasm areas (mean values) are reported as histogram for each experimental category,

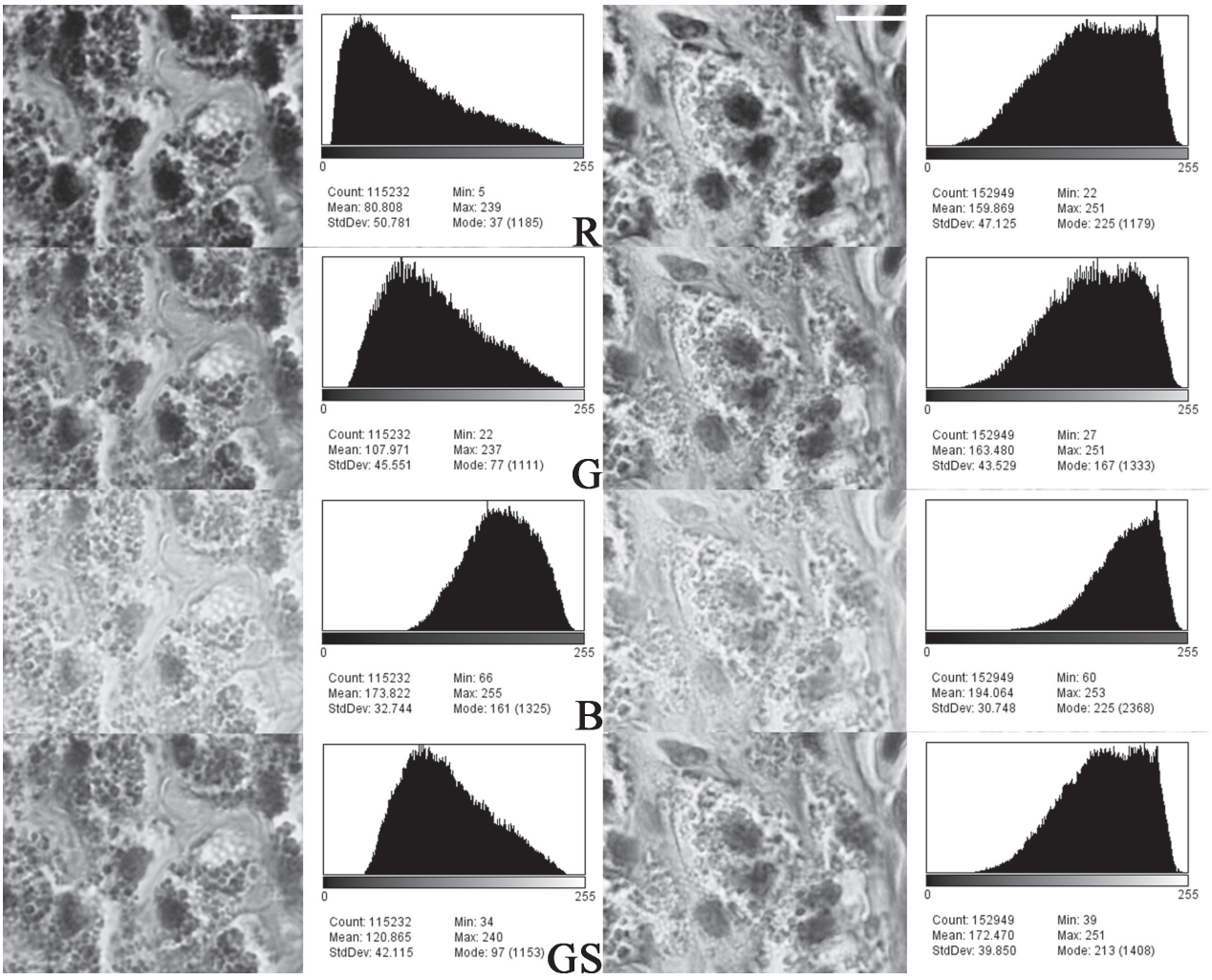

Figure 1. Rainbow trout. *Stratum granulosum*. Clusters of mast cells from an untreated (on the left) and compound 48/80 treated (on the right) intestinal strip according to RGB channel are appreciable. Red (R), Green (G), Blue (B) stacks equivalent images and eight bit gray level image (GS). Mean gray values of five randomly selected granular cytoplasm areas are reported as histograms for each section (on the right side) and for each experimental category (before and after compound 48/80 addition). Differences of mean gray values are clearly appreciable according to treatment, in particular in Red and Green channels. Giemsa's stain. Scale bar= 10 μm.

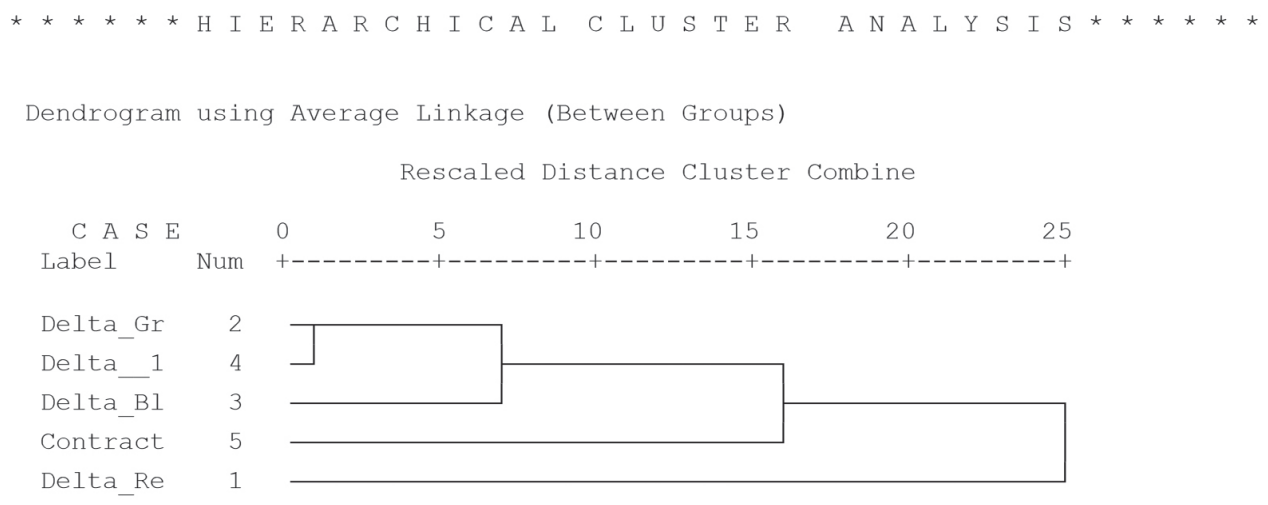

Figure 2. Hierarchical cluster analysis. Euclidean quadratic distance (mean linkage method) Values are standardized according to variables (Z scores). Distances among variables are clearly appreciable. Delta_Gr = Δ Green, Delta_1 = Δ Grayscale, Delta_Bl = Δ Blue, Contract = Contraction, Delta_Re = Δ Red.

in order to summarise graphically the differences in optic density, according to RGB channels.

With regard to partial correlation, Δ mean gray value of the Red channel did not correlate with the respective value of maximal intestinal contraction. On the contrary, Δ mean gray value of Green (R^2 0.84, $P < 0.01$), Blue (R^2 0.83, $P < 0.01$) channels and eight bits grayscale (R^2 0.76, $P < 0.05$) correlated significantly with the respective value of maximal intestinal contraction (12.7 ± 0.6 g cm^{-1}; mean ± standard error). This can be graphically summarised by means of hierarchical cluster analysis, standardizing values according to variables (Z scores) and appreciating the Euclidean distance among the latter, with Δ Green and Δ Blue sharing the same distance from contraction variable and Δ Red showing the highest distance (Figure 2).

Details on intestine contractile dose-response to compound 48/80 administration have been reported elsewhere (MANERA, GIAMMARINO, BORRECA et al., 2011).

4 Conclusion

Results show that intestinal contraction in trout correlates with degranulation intensity of mast cells assessed by means of gray-level analysis, with particular regard to Green and Blue channels, which provide better correlation compared to eight bit grayscale. On the contrary, Red channel mean gray values do not correlate with intestinal contraction. The loss of information in grayscale images is known and use of three monochrome images, Red, Green and Blue, namely RGB color representation, has already been applied to histopathological morphometry and densitometry (SHARIPO, HARTANTO and LEPOR, 1992; SERTEL, KONG, CATALYUREK et al., 2009; MANERA and BORRECA, 2012). Moreover, the usefulness of mean gray values analysis of the three RGB channels to discriminate degranulation of MCs has been recently reported (MANERA and BORRECA, 2012).

Color images are produced by color **CCD** (Charged-Coupled Device) cameras, in which color filter arrays (Bayer mask) are placed over the image sensor. RGB is the most used color space, describing the latter the gamut of colors that image handling devices deal with (GUNTURK, GLOTZBACH, ALTUNBASAK et al., 2005; FERREIRA and RASBAND, 2011). In particular, Green channel is measured at a higher sampling rate because the Green band corresponds to the sensitivity peak of human vision (GUNTURK, GLOTZBACH, ALTUNBASAK et al., 2005; FERREIRA and RASBAND, 2011). This feature, in conjunction with color to grayscale transformation algorithm, help to elucidate why Green channel mean gray values are very close to eight bits grayscale mean gray values (refer, also, to Euclidean distance in Figure 2).

As stressed by Gunturk, Glotzbach, Altunbasak et al. (2005), CCD is structurally sensible to photons and has a specific spectral response, which is a function of the spectral wavelength, and a spatial response light and is unable to represent color directly. Color filter array acts as a bandpass filter. In particular, red filter passes the red component of the visible spectrum, which tends to white in a grayscale representation, and blocks the green and the blue components, which tends to black in a grayscale representation; green filter passes the green component and blocks the red and the green; blue filter passes the blue component and blocks the red and the green. Color image is then reconstructed through an interpolation process named demosaicking (GUNTURK, GLOTZBACH, ALTUNBASAK et al., 2005).

In the present survey, only Green and Blue channels Δ mean gray values correlated with maximal contraction, suggesting the putative pro-contractile agonist had an affinity to the blue-green component of Giemsa's stain. In effect, granules of degranulated, effete MCs appeared faintly blue or pink (eosinophilic) with Giemsa staining technique, compared to the intensively blue stained (basophile) granules of not degranulated MCs. This phenomenon suggests the putative pro-contractile agonist belongs to the basophile component of the Giemsa stained granular cytoplasm of MCs, which depletes during degranulation process proportionally to maximal intestinal contraction.

Mean gray values analysis allowed discriminating granule density in all the three RGB color channels, which is inversely related to mediators releasing. Similar findings have been previously described, but no attempt to differentiate between color channels, to use partial correlation method and to discriminate histochemically the putative pro-contractile agonist has been done (MANERA, GIAMMARINO, BORRECA et al., 2011).

Giemsa's stain is a solution of polychrome methylene blue and eosin, where the oppositely charged ions combine to form azure eosinate salts, insoluble in water and soluble in alcohols (KIERNAN, 2010). Nowadays, Giemsa's stain solution is a concentrated stock solution of azure B eosinate with methylene blue in alcohols (methanol and glycerol, 1:1), then diluted in water at appropriate pH to obtain the staining solution (SANDERSON, 1994). With regard to Giemsa staining mechanisms, dye cations dimers are attracted by the negative phosphate groups of DNA or other acid molecules. Moreover, there is adherence due to hydrophobic interactions with the purine and pyrimidine rings of the DNA (HOROBIN and WALTER, 1987). Eosin is attracted to proteins with excess protonated amino and guanidino groups (e.g. the amino acids lysine and arginine) compared to ionized carboxyl groups (e.g. glutamic and aspartic acids) (WELLER, ACKERMAN and SMITH, 1988). Furthermore, caution should be paid in the interpretation of "colorimetric" measures provided that uniform staining, image capture and processing conditions have been applied to all the microphotographs as suggested by Benattar and Flandrin (1999) in a morphometric and RGB colorimetric quality control study for May-Grünwald Giemsa stained preparations. During the present survey, particular attention was paid to ensure same staining condition, microscope lighting and digital camera setting, in order to minimize error sources.

Basing upon the aforementioned consideration, a possible acidic/anionic or amphiphilic pro-contractile agonist can be inferred. Anionic constituents in mast cells granules, ascribed to sulfated molecules, have been extensively studied by means of various polycationic probes (SKUTELSKY, SHOICHETMAN and HAMMEL, 1995; HAMMEL et al., 2010). Interestingly, sulphated glycosaminoglycans, in particular heparin, are reported to enhance human uterine strip contractility and a patent application for

establishing effective labor in women has been recently published (EKMAN-ORDEBERG and MALMSTRÖM, 2008; EKMAN-ORDEBERG, HELLGREN, AKERUD et al., 2009; EKMAN-ORDEBERG, AKERUD, DUBICKE et al., 2010). Sulfated glycosaminoglycans and heparin-like components have been reported in fish, though granule metachromasia appeared to be inconstant according to species, fixative and staining methods (REITE, 1998; ROCHA and CHIARINI-GARCIA, 2007).

As remembered earlier, Salmoniformes display large clusters of MCs in the so named *stratum granulosum* (ELLIS, 1977; EZEASOR and STOKOE, 1980; REITE and EVENSEN, 2006). The exact role of the latter is unknown, possibly associated to a first line of defense (REITE, 1997; REITE, 1998; REITE and EVENSEN, 2006). Indeed, due to the morpho-functional relationships with the underlying tunica muscularis described during previous (MANERA, GIAMMARINO, BORRECA et al., 2011; MANERA and BORRECA, 2012) and present surveys the role of mast cells in intestine contractile modulation in trout should be further investigated.

Actually, a possible multi-agonist cascade could be involved as previously suggested by Manera, Giammarino, Borreca et al., (2011). In particular putative basophile agonist may represent the first step of the latter. To date, information on agonist nature involved in compound 48/80-elicited intestinal contraction is lacking (MANERA, GIAMMARINO, BORRECA et al., 2011), though evidences of the involvement of an acidic component, possible glycosaminoglycans, are reported (this survey).

In conclusion, the proposed *ex vivo* morpho-functional animal model and the RGB stacks gray-level analysis represent a possible alternative in mast cells research and a reliable experimental tool to study the role of piscine mast cell in intestine contractile modulation both in physiological and pathological condition.

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