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SHORT COMMUNICATION

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Persistence of Autofluorescence of parathyroid glands submitted to heat, freeze and formalin

Carlos Serra*
Hospital do Sams, Lisboa, Portugal Faculdade de Ciências da Saúde, Universidade da Beira Interior, Covilhã, Portugal

ABSTRACT

Autofluorescence of parathyroid glands is a physical phenomenon of unknown cause that could have clinical impact for intraoperative identification of the glands. Persistence of Autofluorescence after cryopreservation and fixation in formalin was reported by other authors suggesting the robustness of the event. Information about persistence of Autofluorescence after heating, with the consequent protein denaturation is less documented in literature. In this work we analyze the intensity of Autofluorescence of five parathyroid samples submitted to heat, freeze and fixation in formalin. There was no significant difference on the intensity of Autofluorescence of parathyroid glands submitted to neither condition with p-values of 0.82 (heating), 0.075 (freezing) and 0.314 (fixation). Those results exclude the necessity of a chemical reaction as well as the need of the integrity of the secondary and tertiary protein structure for the occurrence of auto luorescence of parathyroid glands. The persistence of Autofluorescence after fixation in formalin facilitates further research in the area, avoiding the need for fresh samples, difficult to obtain and maintain.

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Introduction

Autofluorescence of parathyroid glands when submitted to a 785 nm laser was described for the first time in 2011 by a group of investigators from Vanderbilt University emerging as a potential tool for helping surgeons on the identification of those glands [1]. Parathyroid glands (usually four) are localized in close proximity with the thyroid and their small size (5 mm to 7 mm) and color makes them difficult to distinguish from other cervical tissues like fat nodules, lymph nodes or even small thyroid nodules [2,3]. As the available tools for intraoperative identification of parathyroid glands are limited it still depends mainly of the experience and visual acuity of the surgeon. The initial study of Paras et al. demonstrated that after stimulation with a laser source of 785 nm parathyroid glands emit fluorescence with a peak on the 822 nm wavelength. Thyroid glands also emit fluorescence at the same wavelength, but its lower intensity allows an easy discrimination from parathyroid. Fat and lymph nodes don't emit fluorescence [1]. Autofluorescence of parathyroid glands is a curious phenomenon as there were no known intrinsic fluorophores emitting at this wavelength [4,5]. The responsible fluorophore must exist in both parathyroid and thyroid, but in higher concentration on parathyroid, and be absent in other cervical tissue still remains unknown. Even though unknowing the source of Autofluorescence was not an obstacle to its clinical utilization, that knowledge could expand the applications of the phenomenon. Previous works stated that parathyroid Autofluorescence remains even after fixation of the tissue in formalin so excluding the need of a chemical reaction in vivo for the occurrence [6,7]. The persistence of Autofluorescence of a cryopreserved parathyroid was also reported by Moore et al. in a single case report [8]. These data suggest that Autofluorescence is a robust occurrence, persisting even after exposition to adverse conditions. The

Contact: Carlos Serra caaserra@netcabo.pt Hospital do Sams, Lisboa, Portugal Faculdade de Ciências da Saúde, Universidade da Beira Interior, Covilhã, Portugal

possibility of Autofluorescence being caused by an enzymatic reaction may be excluded by its persistence after submission to a heat source. Heat causes protein denaturation disrupting hydrogen bonds and non-polar hydrophobic interactions thus affecting secondary and tertiary structures. The denaturation process is not strong enough to break peptic bonds so doesn't affect the primary structure (amino acid sequence) [9-14]. The persistence of Autofluorescence after freezing could have application for verification of cryopreserved tissue amenable to auto transplantation in cases of secondary hyperparathyroidism submitted to para thyroidectomy.

Formalin fixation is used for tissue preservation helping to maintain cellular architecture and composition of cells and allowing subsequent processing. Fixation also preserves the proteins, carbohydrate and other bio-active molecules in their spatial relationship to the cell, so that they can be studied [15,16]. In this work we analyze the persistence of Autofluorescence in fresh parathyroid samples submitted to heat, frozen and fixation with formalin. Being a preliminary work, the main objective was to confirm the persistence of Autofluorescence after extreme conditions without using the usual techniques for cryopreservation of tissues.

Methods

Five fresh samples of parathyroid adenomas were divided in 3 fragments (fragments A, B, C). After division all samples were observed through a night vision goggles coupled with 822 nm band pass filter while irradiated with a 780 nm LED with a 780 nm band pass filter and submitted to Heat (A), freeze (B) or fixation with 10% formalin (C). The system was coupled with a laptop personal computer through a USB video recording board and image software was used to record the images. Recorded Images were analyzed with Image J software U. S. National Institutes of Health, Bethesda, Maryland, USA). Statistical analysis of data was made with IBM SPSS Statistics V.25 (IBM Corp. Released 2017. IBM Statistics for MacIntosh, Version 22.0. Armonk, NY: IBM Corp). For data with a normal distribution evaluated with Kolmogorov-Smirnov and Shapiro Wilk tests a parametric test was used (Student's t test and ANOVA). When normality was violated non-parametric tests were used (Kruskall Wallis and Friedman). A level of significance of 0.05 was considered significant.

Heating

Fragments of group A were submitted to a temperature of 80° for 30 minutes through a Bio-Optica

WB-100 I (Bio-Optica Sp, Milan, Italy) water bath and immediately observed with the near infrared device. 24 hours and 48 hours after heating tissues were observed again.

Freezing

Fragments of group B were frozen at 50° in an ultra-low temperature freezer Herafreeze HFU (Thermo Fisher Scientifics, Waltham, MA, USA) and observed with the device. They were defrosted after 30 days and observed. 24 hours after defrosting another observation was done.

Fixation

Fragments of group C were emerged in a solution of 10% neutral buffered formalin and observed after 2, 15 and 60 days.

Results

Heating

Intensity of fluorescence (arbitrary values) measured by Image software are shown in (Table 1).

As normality conditions were not achieved ($p<0.05$ on Kolmogorov-Smirnov and Shapiro-wilk tests) for values collected 24 hours after heating Friedman test was used. There were no significant differences between groups (Friedman Test value 1.125) $p=0.818$.

Freezing

Intensity of fluorescence (arbitrary values) measured by image J software are shown in (Table 2).

The normal distribution of data allowed the utilization of a parametric test (ANOVA for repeated measures).

No significant difference was obtained between groups ($F=2.959$) $P=0.075$.

Fixation

Intensity of fluorescence (arbitrary values) measured by image J software are shown in (Table 3).

Data had a normal distribution allowing utilization of ANOVA test for repeated measures. There was also no significant difference between groups (F=1.345) p=0.314. No Post Hoc comparisons were done were done since there were no differences between groups.

	Before heating	After heating	24 hours	48 hours
1	247	249	247	249
2	246	253	247	247
3	246	245	249	247
4	247	246	248	246
5	249	247	249	249
Mean (sd)	247,6 (1.34)	248 (3.16)	248.2 (1.10)	247.6 (1.34)

Table 1. Intensity of Autofluorescence of Heated Parathyroid Glands

	Before freezing	After freezing	24 Hours	48 Hours
1	247	213	247	247
2	246	243	246	247
3	247	249	249	245
4	246	238	245	245
5	248	237	248	248
Mean (Sd)	246.8 (0.84)	236.0 (13.71)	247.0 (1.58)	246.0 (1.34)

Table 2. Intensity of Autofluorescence of Frozen Parathyroid Glands

	Before fixation	48 Hours after	180 days
1	247	246	245
2	246	246	247
3	247	247	248
4	248	243	246
5	248	247	245
Mean (sd)	246.2 (0.84)	245.8 (1.64)	246.2 (1.30)

Table 3. Intensity of Autofluorescence of Parathyroid glands Fixed in Formalin

Discussion

The results of this work reinforce the robustness of parathyroid glands Autofluorescence. In fact, persistence of Autofluorescence after exposition to extreme conditions excludes the need for any kind of chemical reaction for its occurrence as it would have been affected by the heat. The phenomenon is also independent of the maintenance of the aeroespacial arrangement of the proteins since modification of the secondary and tertiary structure doesn't affect it. Those results confirmed that the probable responsibility initially assigned to the Calcium Sensing receptors or to Vitamin D receptors can almost be excluded [4]. The values obtained after freezing and defrosting also confirmed the results of Moore et al. that described the persistence of Autofluorescence in a case of cryopreserved gland [8]. Even though we hadn't used the methodology of cryopreservation, the preservation of Autofluorescence for a long period of time was well documented. The small size of and the nature of sample, composed only of parathyroid adenoma fragments, don't analyzing normal parathyroid tissue, unavailable for ethical reasons, is a strong bias of this study in spite of being almost consensual that the intensity of Autofluorescence of normal parathyroid is higher than the Autofluorescence of abnormal tissue [17]. The persistence of Autofluorescence after cryopreservation opens a field of research on the relation of the intensity of the fluorescence and the viability of the transplanted glands that could have clinical application. That research is however very difficult to build since few surgical centers all over the world have significant auto transplantation casuistic. Our study could also confirm the initial observation of Kim et al. of the persistence of Autofluorescence after fixation in formalin [7]. That observation, although without clinical impact, could be important for the development of new devices decreasing the dependence of fresh parathyroid tissue that is always difficult to obtain and maintain allowing research with conserved tissues, easier to achieve, with the same accuracy.

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