Staining of the Reinke Crystalloids in the Human Testis – Re-evaluation Study

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Summary: Reinke crystalloid (CR)s are rod or corn shaped structures present in the interstitial cells (Leydig cell) of human testis. Due to existing controversies and because of their importance in histological studies, we decided to re-evaluate their staining behavior with various dyes. Earlier it has been shown that hematoxylin and eosin dyes do not stain CRs even though their protein nature remains undisputed. In the present study, sections of testicular tissues embedded in glycol-methacrylate (GMA) showed that hematoxylin binds to CRs non-specifically and eosin stains them specifically. The reasons for stainability and/or non-stainability of CRs with hematoxylin, eosin and other dyes are discussed.

In 1896, Fr. Reinke reported the rod or corn shaped CRs structures measuring up to 20 μm in the interstitial cells (Leydig’s cells) of human testis¹). Later, these structures were named after Fr. Reinke, Reinke CR. In 1912, Winiwarter found smaller rice shaped CRs in iron hematoxylin staining preparations and hypothesized a possibility of aggregation of these rice shaped CRs to form huge CR²). Electron microscopically, the presence of CR was also confirmed³–⁵). However, till date, CRs are specific to only human tissues. Reinke described proteinacious nature of CR and stained them with fuchsin, iodine, safranin, and iron hematoxylin¹). Recently, eosin was reported as an effective and specific dye for protein analysis⁶). However, it has been reported that the CRs are not stained with eosin dyes⁷–⁹)

In the present study the CRs were stained with eosin in GMA embedded material. The reasons of the stainability of the CR with eosin dye are discussed and reasoned out why the earlier authors were unable to stain CR with hematoxylin and eosin (HE).

Materials and Method

I. Fixation and Embedding

Human testes removed from prostate cancer patients (50 to 70 years) who were on hormone therapy, were used in this study. The testes were fixed for 12 hrs in 2.5% glutaraldehyde adjusted to pH 7.4 with 0.05 M cacodylate buffer. The specimen was washed with the same buffer and dehydrated routinely with alcohol, and embedded in glycol-methacrylate (GMA) (Technovit 7100, Kulzer). The specimen was sectioned at 4 μm thickness on a Sorvall JB-4 microtome fitted with Ralph’s glass knife¹⁰), dried on glass slide, and stained with different procedures on the same section. After staining, the sections were mounted using Entellan neu (Merck) mounting medium, photographed and then cover glass was removed in xylene, the section was again stained with another dye. Olympus light microscope (Vanox and BX50) was used for the examination and photography.

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2. Staining

In this study, only GMA embedded sections without removal of GMA were used.

HE staining

1) Carazzi's hematoxylin\(^{11}\) modified (hematoxylin \(\times 2\)) staining: Sections were stained with the modified solution for 24 hours.

Modified solution:

- Hematoxylin 2 g
- Aluminim potassium sulfate 50 g
- Sodium iodate 0.4 g
- Distilled water 200 ml
- Glycerin 200 ml

2) Routine eosin staining: Following the hematoxylin staining modified method, 1\% eosin Y, and a mixture of 95\% ethanol which includes 1\% phloxine B and acetic acid was used.

3) Single eosin staining: Aqueous solutions of 1\% phloxine B, 1\% eosin Y, 1\% eosin B, and alcohol soluble eosin (1\%) were used to counter stain the specimen which were stained with Carazzi's hematoxylin earlier.

Periodic acid methenamine silver (PAM) stain\(^{12}\)

After viewing the PAM stained specimen, the same section was stained with eosin and investigated for the possibility of multiple staining of CRs.

In another specimen, after HE staining, PAM was done to differentiate the intracellular organelles.

Toluidin-blue\(^{13}\), light green\(^{14}\) and Heidenhain iron hematoxylin stains\(^{15}\) were also used.

Results and Discussion

In addition to Reinke CRs, often CRs of Lubarsch and Charcot-Bottcher\(^{16}\) were also reported in the epithelial cells of testis. They are of variable sizes and thought to be the storehouse of proteins. However, yet the functional significance of these CRs is not well understood\(^{17}\). Reinke CRs are crystalline structures and are found in the cytoplasm of human testicular interstitial cells (Leydig cells) which produce testosterone\(^{18}\). The functional significance of these structures is also not clear\(^{19}\). Their stainability with fuchsin, iodine, safranin, and Heidenhain hematoxylin was reported by Reinke\(^{11}\). However, he did not use HE stain. Historically, Stieve in 1930 was the first to report that the CR were not stained with HE and remained transparent\(^{16}\). Since then micro-photographs of CR in most of the histological textbooks and atlases were included from the preparations using Heidenhain iron hematoxylin\(^{20,22}\), or Mallory-Azan stains\(^{15}\). Fujita and Fujita in their Textbook of Histology included negative image of CRs stained with HE\(^{23}\). Detailed electron microscopic account of CRs has been published\(^{7,9,21-24}\).

1. HE stains

Routine for staining GMA sections, hematoxylin and eosin (eosin Y + phloxine B) is used. Eosinophilic nature of CR was established by using routine method (Fig. 1). Further, CRs were also stained by using eosin Y, eosin B, phloxine B, and an alcohol soluble eosin (Figs. 2–5).

Eosin stains

CRs are stained with all kinds of eosin dyes, and among the eosin dyes, especially phloxine B stains deep pink and shows good contrast with the surrounding cytoplasm (Fig. 2). The contrast of CR with eosin Y (Fig. 3) is unclear and inferior to other eosins (Figs. 2, 4, 5). The specimen with eosin B is stained pink (Fig. 4), and the stainability with an alcohol soluble eosin is next to phloxine B (Fig. 5). Eosin dyes have a good affinity to proteins and can bind to very small quantities of proteins\(^6\) and, therefore, CR also showed good affinity with these dyes contrary to the earlier reports.

Earlier, it is explained that crystals of Reinke have little affinity for the common histological stains and, therefore, appear nearly colorless in routine preparations and they can, nevertheless, be recognized as negative image in ordinary preparations\(^7\). There is another possibility of shedding off the CRs from the wax sections and the earlier workers might not be able to stain these structures, even if they are well preserved in the preparations because of less color contrast depends on a kind of the used eosin, these structures were not well documented in the preparations. Moreover, since the CR is not enveloped by the limiting membrane\(^4\), the boundary between the CR and the cytoplasm is unclear. In the previous reports differences of staining between several eosin dyes used were not described. To obtain better contrast between the CR and cytoplasm phloxine dye is recommended based on the present study.

As far as negative image in ordinary methods\(^7\), we think that these images could be the trace or impression of the crystals that may be due to dislocation or drift of CRs while sectioning therefore the CRs are detached in the process of removal of paraffin or epoxy embedding resin. Further, Leeson et al.\(^{20,21}\) indicated a negative image of Reinke CRs which should normally be stained black with iron hematoxylin. This is thought to be a typical case of detachment of CRs. Also if the negative
image of CRs is thought to be the crystal itself, it is necessary to stain the same crystal with other dye which stains the crystals positive.

In the GMA embedding section, removal of the GMA is not required for staining. It suggests that resin as embedding medium preserves tissue structure and produces much less distortion, because the detachment of cell contents is less probable. In the cross section of CRs embedded in paraffin, the CRs are rather left in the cells than fallen off, but they are difficult to differentiate from the surrounding cytoplasm. On the other hand, in the longitudinal section, it is easy to differentiate the CR from the surrounding tissue, but they fall off easily.

Hematoxylin staining

In single staining with Carazzi’s hematoxylin, the nucleus and ergastoplasm are stained blue basophilically, however, CRs are stained not blue but yellow ochre (Fig. 6A). Since ochre yellow is not the color of hematoxylin staining, the present authors would imagine that this may be a non-specific stain. Following the hematoxylin staining, eosin stains the portion of ochre yellow eosinophilic (Fig. 6B). Oxidized hematoxylin or hematein, which is primarily in negative charge, connects to the base and change in positive to reveal blue color. In other words, Reinke CR conjugates with hematoxylin but does not change its hematein in positive charge.

2. PAM stain

In PAM staining, periodic acid oxidizes polysaccharide to develop aldehyde radicals, to which methenamine-silver complex salt binds. The basement membrane, nucleus and a number of lipochrome pigment are able to stained brown-black in the GMA embedding section (Fig. 6C, 7A), but CRs showed non-specific gray colour (Fig. 7A). These results indicate absence of glycoproteins in the CR. When the PAM stained section is restained with eosin, CRs stained pink (Fig. 7B) and when HE stained section is stained with PAM, enormously different configure of the Leydig cells are revealed. The boundary of cells and CRs are much

Fig. 1. Hematoxylin-EosinY and +PhloxineB staining.
The rod shaped longitudinal structure of Reinke CR stained with eosin dye. Leydig cells are steroid producing cells, therefore, contain a number of lipid droplets (arrows). RBC: red blood cell. ×1,850.
clear, but lipochrome pigments, which are stained brown in the Figure 7, are not differentiated in the Figure 6C. By using multistaining techniques with histochemical staining e.g. PAM and general staining e.g. HE, it is possible to differentiate several intracellular organelles and extra cellular components.

3. Toluidin blue stain

With toluidin blue the CRs are stained light green or clear sky blue with a clear contrast to the basophilic cytoplasm which is thought to be an intracellular ribosome rich area, and do not reveal the metachromasia (Fig. 8). Mori et al. published a micrograph of the Leydig cells which were fixed
Fig. 6. The same section stained with different ways of staining. ×2,000.

A. Modified Carazzi’s Hematoxylin stains the nuclei and cytoplasm specific blue, but stains Reinke CRs non-specific ochre. Lipid droplets and lipochrome pigments (arrows) are not stained.

B. Eosin is added to the specimen of A to make routine HE staining. Reinke CR are stained with eosin dye, but the contrast is poor. Lysosomes in the cytoplasm are stained red (arrows).

C. HE staining + PAM staining.

PAM staining of B. Reinke CRs are showing the silver deposit of PAM staining. Lysosomes (arrows).
glutaraldehyde and osmium tetroxide, embedded in epoxy resin, and stained with toluidin blue, but did not mention staining nature of CR. The CRs and cytoplasm in the cell were stained so darkly that the measurements of CRs and other cell organelles were done in section stained with Heidenhain iron hematoxylin after removal of the epoxy embedding resin.\(^2\) The staining property of CRs is similar to that of red blood cell (Fig. 8).

4. **Light green stain**

The CRs are stained well with a light green staining dye and are clearly visible when stained with hematoxylin prior to light green (Fig. 9).

5. **Heidenhain iron hematoxylin stain**

With Heidenhain iron hematoxylin, the CRs are stained black (Fig. 10). The stainability is same as described earlier.\(^1,2,8\)

**Conclusions**

1. Reinke CRs can be stained with eosin dyes by careful fixation and embedding of the specimens.
2. The CR is stained non-specifically with hematoxylin or PAM staining methods.
3. The staining properties of the CR are similar to that of red blood cells.
4. When the CR is stained with HE, much contrast is not produced between it and the cytoplasm.

5. The negative image of CR may be due to detachment of CR in the processes of sectioning and/or during removal of embedding medium.

6. The advantages of GMA usage as embedding medium lies in its properties to preserve cell structure and to be able to stain sections without removal of the resin at least for such cellular structures which may fall off during tissue processing.

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