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APPLICATIONS OF IMMUNOHISTOCHEMISTRY TECHNIQUE ON FORMALIN-FIXED PARAFFIN EMBEDDED RAT'S IMMUNE TISSUES AFTER TREATMENT WITH VINCRISTINE SULFATE

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ABSTRACT
The present study was designed in order to explain the details application of Immunohistochemistry technique on formalin-fixed paraffin embedded rats' immune tissue after treatment with Vincristine sulfate. 15 rats were used and subdivided in 3 group each one contain 5 rats, 1st one immunized with specific antigen (Brucella vaccine), 2nd one non immunized and third one control treated with distal water. Vincristine sulfate injected at dose (0.1mg/10 gm body weight) intraperitoneal for one month at weekly dose. Rats were anesthetics at the end of experiment, tissue samples from lymph node and spleen tissues were took, so that to detection level of lymphoid marker (CD^4 CD^8). Finally we can conclude that Vincristine caused depression in cellular immunity by estimation the percent of lymphoid marker (CD^4, CD^8).

KEY WORDS: Immunohistochemistry, rat, CD^4 CD^8, Vincristine.

INTRODUCTION

Immunohistochemistry (IHC)

Immunohistochemistry its' a technique to demonstrate T-cell and T-cell subsets informal in fixed, paraffin embedded lymphoid tissues as spleen , lymph node , and payer's patchy immunotoxicity is an important cause of drug-related adverse events, contributing to the withdrawal of a number of drugs from the market in the past 15 years, over recent years a number of proposal have been made in an attempt to rationalize and improve Histopathological assessment of immune system (Kupere *et al*., 2000; Kupere *et al*., 2002; Germole *et al*., 2004; Kevin, 2008; Jerrold *et al*., 2012). Immunohistochemical staining is accomplished with antibodies that recognize the target protein. Since antibodies are highly specific, the antibody will bind only to the protein of interest in the tissue section. The antibody-antigen interaction is then visualized using either chromomeric detection, in which an enzyme conjugated to the antibody cleaves a substrate to produce a colored precipitate at the location of the protein, or fluorescent detection, in which a fluorophore is conjugated to the antibody and can be visualized using fluorescence microscopy (Robert *et al*., 2011; Janeway *et al*., 2005; Haines,1991; Van, 1981). IHC-P refers to the staining of tissues that have been fixed (usually in neutral buffered formalin) and then embedded in paraffin before being sectioned (Haines, 1991). The initial screen for potential immunotoxicity involves standard Toxicity studies, including of a recommendation for the semi quantitative description of change in the separate compartments of lymphoid tissue in sections stained routinely for morphology immumophenotype identification / enumeration of Leukocyte subset, conducted using either flowcytometry or IHC-P (Van, 1981) Is suspected from the standard studies some immunotoxicants, such as Vincristine sulfate can induce depletion of subset of T-cells (Jerrold *et al*., 2012) prior to exhibiting more generalized lymphocyte toxicity at a higher dose. The detection and counting of shifts in T-cell population distribution in preclinical studies is usually performed by flow cytometry on peripheral blood or disaggregated tissue or occasionally by Immunoflorescence on section organs (Janeway *et al*., 2005). Their techniques have over require specialty preparative produce with prospective sample collection and connected be carried out retrospectively the success of IHC depends on the interaction between the primary antibody and tissue antigen (Kevin, 2008; Haines, 1991), and any think disrupting this intersection may have detrimental effected on the quality of staining. Historically the Immunohistochemical demonstration tissue antigen has been performed on frozen tissue sections (Van, 1981; Paterson *et al*., 1987; Liou, 1996; Nano, 2000). But the preservation of antigenic epitopes comes at the expense of morphology and Frozen section may suffer from art factional streaming of target sites (Gonza'lez, 2001; Fee, 1996). Aldehyde-based fixatives, such, buffered formalin are favored for preparation of tissue for routine histology and for IHC fixation in buffered formalin result in excellent morphological preservation but may decrease the antigencity of the tissue, possibly because of the protein cross-links created during eldehyde fixation (Cattorate, 1994) or perhaps because of high complexion calcium ions or other divalent metal cat ions with proteins during formaldehyde tissue fixation masking certain antigens(Morgan, 1994). Epitopes unmasking techniques offer no guarantees and the optimal procedure must be determined for each individual antibody (Shi ,1996).

MATERIAL & METHODS

Animals

Fifteen (15) rats were kept in veterinary medicine animals house, and fed on special pellets and drank on tap water

with special bottles. Vincristine was injected in dose(1mg/kg b.w) (Upamanyu, 2011) to rats intraperitoneal at end of each week for four week (one month). Animals divided in three group:1st group injected with Vincristine only, 2nd one injected with Vincristine and Brucella vaccine subcutaneously in dose (0.8ml/rat) according to (Al-Lammy, 2009) Halothane (0.01) was used to anesthesia. Formalin fixed tissue sample were subjected to the routine tissue processing (Kiernan, 2012).

Special kits

Kits Name	Component
Mouse anti – Rats CD4 C2255-04D 1-	Anti-CD4 surface glycoprotein (1mg/ml) Concentration. No dilution was done. stored at -20ºC
Mouse anti – Rats CD8 C2259-36 2-	Anti –CD8 Surface glycoprotein (1mg/ml) Concentration. No dilution was done. stored at -20ºC
Immunohistochemistry Detection Kit 3-	17506A normal serum 1.25 ml 17506B IgG (Biotin) 1.25 ml 17506C solution A 1.3 ml 17506 D Solution B 1.3 ml 17506 E DAB Buffer 50ml 17506 F DAB 1.25 ml 17506 G DAB Detoxification Reagent 5ml Stored at 4ºC

Immunohistochemistry procedure

The laboratory preparing for Immunohistochemical (IHC) Study has endeavored to assemble a tool Kit of IHC stains to identify immune cells population in routinely prepared formalin-fixed paraffin embedded (FFPE) tissue sections. Four µm thick sections from FFPE blocks of rats' spleens were made on positively charged slides and subjected to Immunohistochemical staining procedure according to the protocol of the manufacturing company (US Biological).

Day (1)

1. Slides were immersed in xylene twice, each time for 15 min.
2. Slides were rehydrated in the graded series of ethanol 100% (I), 100% (II) 95%,80 % and 70 % for 5 minute each then incubated in water bath for 5min.
3. Slides were immersed in 0.3 % H2O2 (in distilled water) for 30 min at R.T
4. Slides were rinsed with water and then with P.B.S.
5. A circle was made on each tissue section with a pap pen.
6. Slides were incubated with 1% Normal Serum/P.B.S. The latter was made by mixing 3.5ml of 1X PBS with 35µl of 1706A (normal serum) in a microfuge tube for 30 min at R.T
7. Slides were dropped off the normal serum.
8. Slides were incubated with antibody. This was made by diluting 1µl of antibody with 0.5 ml P.B.S.

Day (2)

9. Slides were washed in PBS three times for 5min each.
10. Slides were incubated with diluted 17506 B anti-mouse IgG (Biotin) made by mixing 35 µl of this secondary antibody with 1.4ml PBS at R.T.
11. Slides were washed three times with PBS for 5min each.
12. Detection Solution was prepared by mixing 1.33 ml of P.B.S with 35µl of 17506C solution A and 35 µl of 17506D solution B in an eppendorff tube and then incubating the mixture at R.T for 30 minute before use.
13. Detection solution was added onto the slides and incubated at RT.
14. Slides were washed three times with PBS for 5min each.
15. Development solution was made by mixing 1.6ml 17506E DAB buffer and 35µl of 17506F DAB in an eppendorff tube.
16. The development solution was added to slides and developed for 30min.
17. Slides were soaked in water.
18. Slides were stained with Harries' Hematoxyline.
19. Slider were Soaked in graded series of alcohol 70%, 80%, 90% 95%, 100% (I) and 100% (II), 3min each.
20. Slides were immersed in Xylene I and Xylen II.
21. Slides were mounted with DPX.

Scoring: IHC result was done by counting the number of CD4 and CD8 marker expression over each lymphocyte of 10 high power fields (oil immersion X1000). The percentage of each was assigned to one of the following scores: score1= (1-10%), score2 = (11-25%), score3= (26-50%). (Laurent *et al*., 2011; Histoch, 2012).

RESULTS & DISCUSSION

Results of scoring

CD^4 and CD8 expression was detected in lymphoid tissue (spleen) of 15 rats in the 1st experimental (non immunized) group, and in 2nd experimental (immunized) group as well as control and results were showed in table (1) and table (2).

TABLE 1:CD^4 percent in 1st experimental group of rats treated with Vincristine only (non immunized) and in 2nd experimental group treated with vincristine and specific antigen (immunized) as well as in control

Group-/Treatment N=5	Score 1% (rats)	Score2% (rats)	Score 3% (rats)
1- 1st group (non immunized)	20%(1) A	20%(1) A	60%(3) A
2-1 2nd group (immunized)	40%(2) A	40%(2) A	20%(1) A
3- control (distal water)	100%(5) A	0 A	0 A

Capital letter denote that significant differences between rats in each treatment ($p < 0.05$)

TABLE 2:CD^8 percent in 1st experimental group treated with Vincristine only (non immunized) and in 2nd experimental group treated with vincristine and specific antigen (immunized) as well as in control

Group-/Treatment N=5	Score 1% (rats)	Score2% (rats)	Score 3% (rats)
1- 1st group(non immunized)	20%(1) A	60%(3) A	20%(1) A
2-1 2nd group(immunized)	40% (2) A	20%(1) A	40% (2) A
3- control(distal water)	5) (100% A	0 A	0 A

Capital letter denote that significant differences between rats in each treatment ($p < 0.05$).

Microscopically Appearances

Pictures of CD^4 CD^8 marker , were took during calculation the number of CD4 and CD8 marker expression over each lymphocyte in 10 high power fields, under light microscopy by(oil immersion X1000).As show in figures(1, 2, 3).

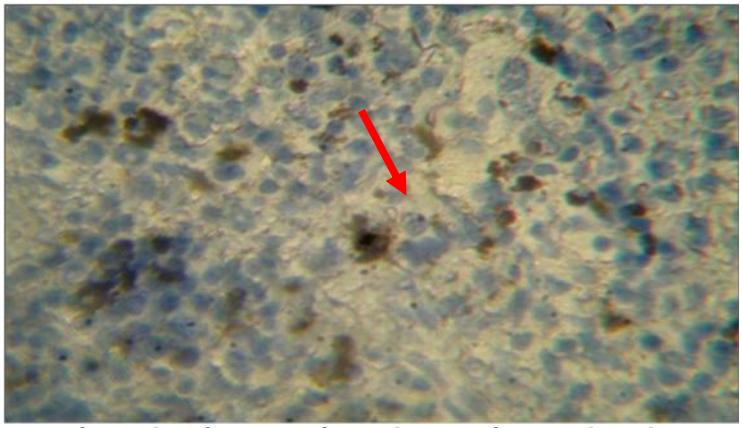

FIGURE1: Score 1: A section from spleen from a rat after application of Immunohistochemistry methods showing CD^4 and CD^8 marker appear, as small dot on lymphocytes (arrow). (Oil.X1000)

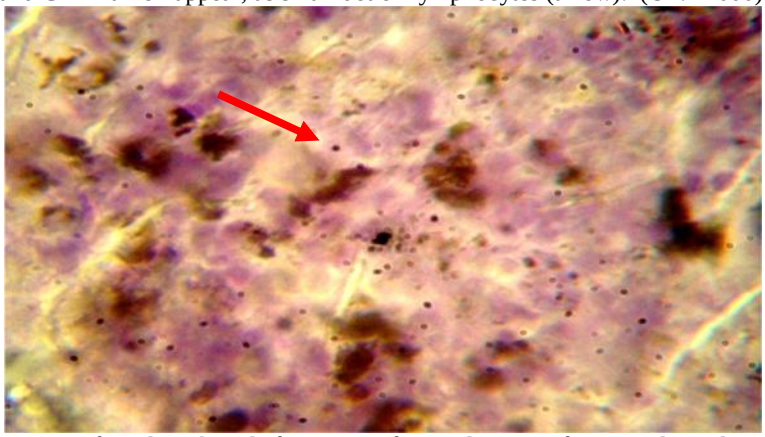

FIGURE 2: Score 2: A section from lymph node from a rat after application of Immunohistochemistry methods showing CD^4 and CD^8 marker , appear as small dot on lymphocytes (arrow). (Oil.X1000)

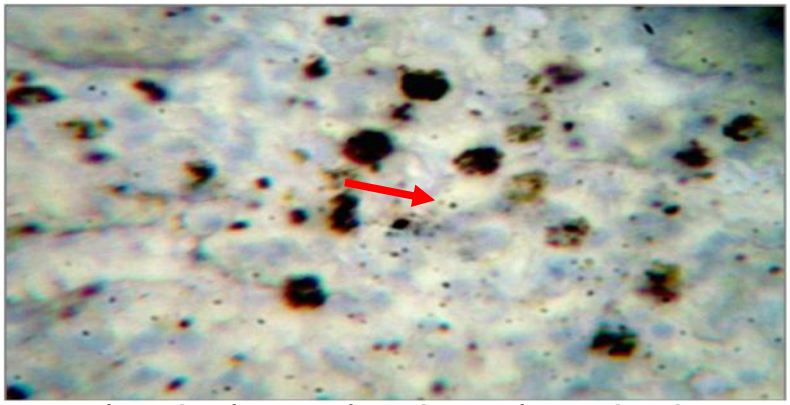

FIGURE 3: Score **3**: A section from spleen from a rat after application of Immunohistochemistry methods showing CD^4 and CD^8 marker appear as small dot on lymphocytes (arrow). (Oil.X1000)

Vincristine is member of chemotherapeutic agents which are considered as potential immunosupressor that indicate poorly immune parameters and interfere with most proliferation reaction that facilitated secretion of immunomodulator like cytokines as INF-γ and interleukins like (IL6, IL12). This interaction due to directed action on proliferators and development machines of immune cell that's' produced antibody as IgM and/ or T-cell which is responsible for producing CD4, CD8 (*i.e.*) proteins lymphoid marker. (Ahmed, 1989; Ageitos, 1999). The results of immunosupressor effects of VCR on cellular immunity agreed with (Pizzo, 1991; Komada, 1992; Lanfranch, 1992; Mackall, 1994; Magrath, 1998; Fuksz, 1998) who explain the role of VCR in induced immune depressions effects by induce T-cell sever depletion with CD4 depletion generally more severe than CD8 depletion and reveal that the most common infection complications associated with cytotoxic VCR treatment are bacterial infections which occur in the setting of neutropenia due to alteration of host defense (e.g. phagocytes cells, lymphocytes, Humoral defense, natural killer cells and skin / mucosal barriers)to specific infections are impaired that contributed to T-cell immune deficient as well as Humoral immune cells. The results of CD4 and CD8 reduction agreed with (Masurtl, 1989; Hernberg *et al*., 1996; Mackall, 1997; Inok, 1997) who showed that VCR treatment as a chemotherapeutic agent resulted in early decrease in CD4:CD8 ratio within few hours-day and revealed that CD4 T-cells dropped from a mean of 588 ±76 / mm^3 before chemotherapy to 105 ± 28/mm^3 (p=0.0002) and CD8 T-cells dropped from a mean of 382 ± 41/ mm^3 to150 ±46/ mm^3 during chemotherapy using flow cytometry methods. Our conclusion that's results of this presence study on immune tissue after application of Immunohistochemistry agreed with these researcher's results.

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