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Impact of fixations on anti-genetic determinants of CD4, CD68, and Pax8 immunohistochemistry: A comparative study of NBF, Zink formalin, and Bouin's fluid

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Abstract

Fixation inhibits autolysis and is an essential step in disease. Our work examined how neutral buffer NBF (NBF), zinc formalin (ZF), and Bouin's fluid (BF) affected tissue specific CD4, CD68, and Pax8 anti-genetic determinants. Fifty-one sections were analyzed for each fixative marker. One hundred fifty-three portions were scored 0, 1, 2, and 3. The comparison between scores in the detection of CD4 epitopes reflects that BF fluid was significant compared to NBF and ZF at a level of $p< 0.05$, whereas NBF comes after ZF. Surprisingly, ZF has no significant scores in the context of CD4 detection. On the other hand, CD68 detection at the score one level revealed that NBF and ZF showed significant CD68 anti-genetic determinants, whereas none of the CD68 epitopes were significantly determined in BF fluid. Eventually, both NBF and BF produced an excellent determination of Pax8 epitopes in contrast to ZF. However, there is no significant difference between them. Regarding scores three comparisons in all fixatives, BF fluid showed high-quality CD4 marker expression in contrast to NBF and ZF; however, neither CD68 nor Pax8 showed any significance in this context. We conclude that NBF showed an excellent quality result in detecting all epitopes. However, BF fluid was supreme in detecting anti-genetic determinants of CD4 and Pax8 epitopes in score 3. Additionally, ZF could distinguish CD68 and Pax8 but failed to detect CD4. Conversely, no significant differences were recorded between both B and NBF.

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Introduction

Fixation is a crucial initial stage in the pathology field, inhibiting the process of autolysis and tissue disintegration (1). In the last two decades, many research laboratories have attempted to substitute NBF with alternative fixatives with lower toxicity (2). However, these endeavors have yielded poor outcomes mostly due to the observed modifications in both cellular structure and antigenicity (3). While each fixative possesses certain advantages, it is important to acknowledge that numerous limitations are also associated with their use (4). Several challenges arise in molecular studies conducted on fixed tissues, such as the degradation of DNA and RNA (5). Furthermore, the ability to maintain the structures of cellular organelles can vary, leading to differences in the characteristics of histochemical and immunohistochemical staining (6). The challenges are associated with using different fixatives for their many purposes and multiple parameters, including size, temperature, pH, and osmolarity, which directly influence the fixation process (7,8) Due to restricted access to epitopes in commonly NBF-fixed paraffin-embedded (FFPE) tissue slices and inadequate amounts of target proteins, cell surface determinants are difficult to determine by immunohistochemistry (IHC) (8), so utilizing Zink NBF treatment on tissue is more effective in identifying a wide

range of cell surface markers (9). This process effectively restricts cellular deformation and the subsequent loss of chemical activity inside the tissue and preservation of cellular antigenicity, thereby facilitating outstanding sample integrity (10). The presence of fixation in ZBF did not result in any practical obstacles during the performance of common laboratory tests in a pathological setting. In each form of fixation, the tissues were adequately NBF, a widely applied fixative in pathology laboratories, which can establish cross-links with proteins dehydrated and deemed to possess comparable characteristics concerning the sectioning of paraffin-blocked and frozen samples (11). The latest modifications of standards for assessing the impacts of chemicals on reproduction advocate (BF) or a "similar fixative" as an alternative to NBF to maintain the architectural quality of histopathological assessment (12). Using Bouin's fixed tissues may not be appropriate when considering later immunohistochemistry (IHC) and structure analyses. It is vital to know the appropriateness of antibodies for use in Bouin's fixed specimens (13). Immunohistochemistry (IHC) is a common pathology technique used to assess biomarkers' presence and distribution inside tissue samples (14). Its primary purpose is to assist in the differential diagnosis and categorization of cancer and other disorders, such as infections (15). Pathological archives contain substantial tissue specimens preserved in NBF and embedded in paraffin (16). These specimens are primarily utilized for medical diagnosis but also serve as valuable resources for scientific endeavors. Heat-induced antigen retrieval facilitates the utilization of tissue specimens for comprehensive immunohistochemistry examination (17). However, it should be noted that antigen retrieval could not fully restore all determinants because of modifications in the original protein architecture caused by NBF (4). Three IHC markers were chosen to measure the influences of different fixatives in this evaluation, which are PAX8, CD4, and CD68. The comprehensive results also validated NBF as a suitable fixative for histology and IHC. Furthermore, additional fixatives based on aldehydes also demonstrate sufficient efficacy (4) PAX8, identified as Paired Box gene 8, is a transcription factor of significant importance in the growth of embryos. The expression of PAX8 can be distinguished in cells of the renal tubular epithelium and is commonly employed as a reliable indicator in the determination of renal cell carcinoma. Distinguishing renal cell carcinoma from other types of kidney cancers can be beneficial (18). Cluster of Differentiation (CD) components are cell surface proteins on leukocytes and other cells that play a significant role in the immune system. According to Engel *et al.* (19), CD4 is a glycoprotein mostly located on the cellular membrane of specific cells, particularly helper T cells. Recently, there has been a growing recognition of these techniques' significant value in treating various cancers and autoimmune-related conditions (19). In contemporary times, utilizing the standard archival tissue format known as NBF-fixed paraffin-embedded (FFPE) is more advantageous for determining the CD4 marking than frozen sections. This preference is mostly attributed to the greater accessibility of facilities that support the FFPE format (20). The signal specific to CD68 exhibited superior performance when using other fixatives compared to NBF. However, it is worth noting that all fixatives led to a higher level of background staining compared to NBF fixation (2). IHCs identify and localize antigens inside tissue sections through immunological and chemical interactions (21). The technique exhibits high sensitivity and specificity, effectively identifying and quantifying a diverse range of antigens across various animal species (2).

The present investigation has been done at the University of Mosul, College of Veterinary Medicine in assessing these three fixatives, neutral buffer NBF, Zink NBF, and BF solution, to recover the antigenic determinant and get the ideal immunohistochemical staining following the specified protocol of each.

Materials and methods

Ethical approve

The University of Mosul College of Veterinary Medicine Administrative Animal Care and Use Council approved experiments on animals (UM.VET.2023.058, January 9, 2024).

Samples

In the summer of 2023,45 samples were engaged in this study, and five rats were euthanized for experiments; these samples were collected from each of the following organs: liver, kidney, and lymph nodes.

Fixative solution used

This study used three fixatives. The samples were collected and immersed in 10% Neutral-Buffered NBF,10 % Zinc NBF, and BF solution for 24 hrs. at 25ºC.

Tissue processing and sectioning

The collected samples go through a series of steps for preparation. First, they were fixed with a 10% (NBF), 10% (ZF), and (BF) solution for 24 hours. Next, the samples were dehydrated using a sequence of ethyl alcohol concentrations: 70% overnight, followed by 80% for two hours, 90% for two hours, and 100% for two hours in two changes. The tissues were then cleared using xylene for 5 minutes in two changes. After that, the samples were transferred to hot paraffin wax at 55-58ºC for one and a half hours with two changes. Once the paraffin block was completed, a rotary microtome with a thickness of 5 µm was used (2). Finally, the slides were left overnight and were ready for immunohistochemistry examination (22-25).

Immunohistochemistry analysis

Three IHC markers, CD4, CD68, and Pax8, were chosen to be identified by specific antibodies for each. Different organs are represented by their particular cell markers, and such markers are later assessed with different fixatives. Liver: is targeted by CD68, found in the cytoplasmic granules. It is particularly useful as a marker for several macrophage lineage cells, such as histiocytes, giant cells, monocytes, osteoclasts, and Kupffer cells (26). The kidney-selected marker was PAX8, housed in epithelial cells of all parts of renal tubules and the parietal cells of Bowman's capsule in the adult kidney (18). Finally, the lymph nodes are assessed with the CD4 marker found on the surface of T helper cells, macrophages, dendritic cells, and monocytes (27,28). Table 1 shows the IHC markers that used in this study.

Table 1: IHC markers details

Marker	Dilution	Target Cell	Catalogue No.
CD68	1:200	Kupffer cell	E-AB-15981
CD4	1:200	T cells	E-AB-22098
Pax8	1:50	Renal epithelial	E-AB-10528

Statistical analysis

Both inferential and descriptive statistics were done with JMP Pro 16.1. Significant frequency differences were found in the findings using chi-square analysis. At $P< 0.05$, the findings were important. JMP Pro16.1 Software, SAS Institute Inc., Cary, NC, 2021.

Results

Our study aimed to assess the effects of three well-known fixatives, NBF, ZF, and BF fluid, on the specific immunohistochemistry (IHC) markers used in veterinary histopathological laboratories. We conducted an immunohistochemical analysis of the liver, kidneys, and lymph nodes of rat's organs. Tissue samples were studied to determine the presence of CD68, Pax8, and CD4 expressions following the immersion in NBF, ZF, and BF fluid (Table 1). The scoring system is addressed in table 2. The IHC results of these markers using different well-known fixatives showed statistically significant variance in scoring criteria within scores and between scores among all fixatives, considering the positive staining immunoreactivity and the number of positive cells reflected an immune reactivity. First, we achieved the scoring system from 0 to 3; table 3 below shows the scoring scale.

Figures 1 and 2 represent the T-cell surface glycoprotein CD4 expression with scoring system. Monoclonal antibody at dilution of 1:200 is expressed in T lymphocytes, B cells, and macrophages. Out of seventeen fields of slide sections were examined in each marker within each fixative. So, regarding CD4 positive immunoreactivity, the statistical analysis in figure 3 revealed that score 3 reflects a significant difference at the level of $P<0.05$ with the 0, 1, and 2 scores in BF fluid, while score 2 reflects a substantial difference with score 0 only. Regarding NBF fixative, scores 3 and 2 have only shown a significant variance compared with 0. Surprisingly, ZF has no considerable record within these scores of this marker.

Table 2: Scoring representation

Scores	Representation
0	No positive cells were detected
1	Positive cells < 20 cells
2	20 > Positive cells < 30 cells
3	Positive cells > 30 cells

Table 3: CD4 scoring results

Scoring	0	1	2	3
NBF	0	4	6	7
Zink NBF	2	4	7	4
BF	0	1	4	12

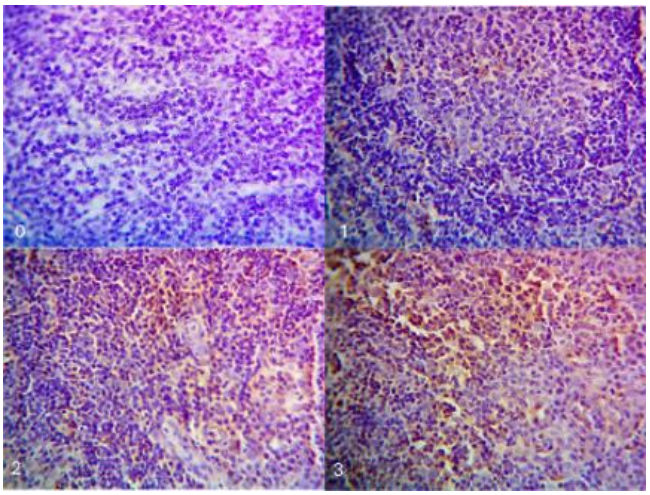

Figure 1: CD4 marker catalog: Upper left (0) represents score zero, upper right represents score one, and lower left represents score two criteria. The lower right panel represents score three. 100X.

CD68

Monoclonal Antibody at dilution of 1:200 CD68 expresses in Kupffer cell. No significant impact was recorded among all scores within BF fluid, while NBF has shown a substantial difference in score one with all scores 0, 2, and 3 and score two compared with score three. Regarding Zink NBF, a significant difference was shown in scores 1 and 2 compared to score 3, as shown in table 4, in addition to figures 4 and 5.

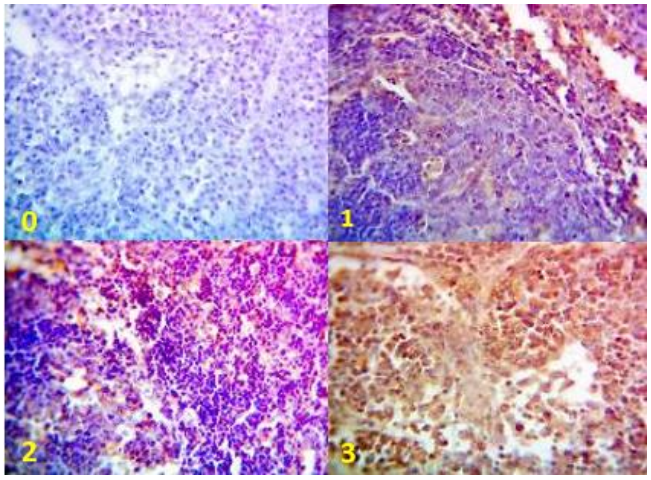

Figure 2: CD4 marker catalog: Upper left (0) represents score zero, upper right represents score one, and lower left represents score two criteria. The lower right panel represents score three. 400X.

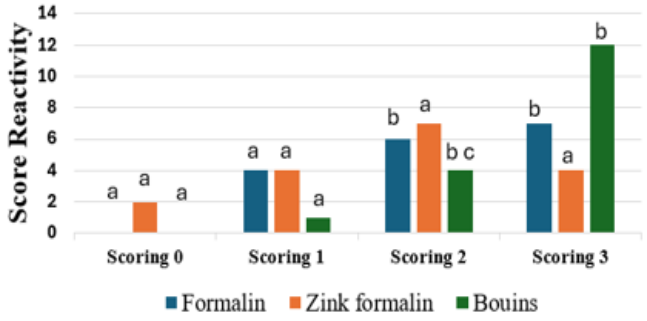

Figure 3: Similar letters specify that there is no significant variance between the fixatives, while different letters indicate that there is a significant difference between the fixatives.

Table 4: CD68 Scoring Data

Scoring	0	1	2	3
NBF	1	12	4	0
Zink NBF	3	7	7	0
BF	3	7	5	2

Pax 8

Monoclonal antibody at dilution of 1:50 of Pax8 marker expresses in renal epithelial cells. This IHC marker in BF fluid showed a significant difference in score three compared with scores 0 and 1. Also, score two shows a difference from score zero. In contrast to the NBF fixative, scores 3 and 2 revealed significances, with scores zero and three compared to scores one. Zink NBF revealed that scores 1 and 2 reflect a significant difference compared to scores zero. The result showed that score three does not affect this marker in comparison with the other scores in the context of this marker (Table 5).

Another statistical analysis was done to compare each score among all fixatives with all IHC markers included in this study; in this context, CD4, CD68, and Pax8 were analyzed statistically among all fixatives, including their scoring criteria 0,1,2, and 3 (Figure 6 and 7). Figure 8 summarizes all results. Intentionally, we only addressed the comparison of score 3 (Figure 9). BF fluid showed a high quality of CD4 marker expression at score 3 in contrast to Zink NBF only at the level of $P<0.05$ in. Surprisingly, neither CD68 nor Pax 8 showed any significance at score three in BF fluid compared to NBF and ZF. At level $P<0.05$, there is no significance within scores 0.1.2 among all markers within all fixatives included.

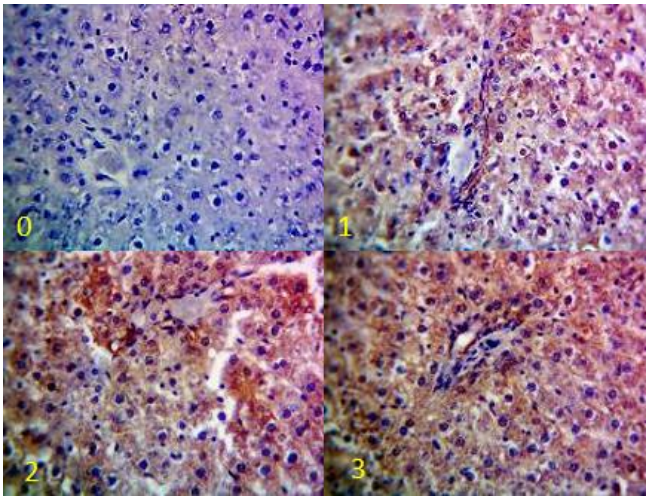

Figure 4: CD68 marker catalog: Upper left (0) represents score zero, upper right represents score one, and lower left represents score two criteria. The lower right panel represents score three. 400X.

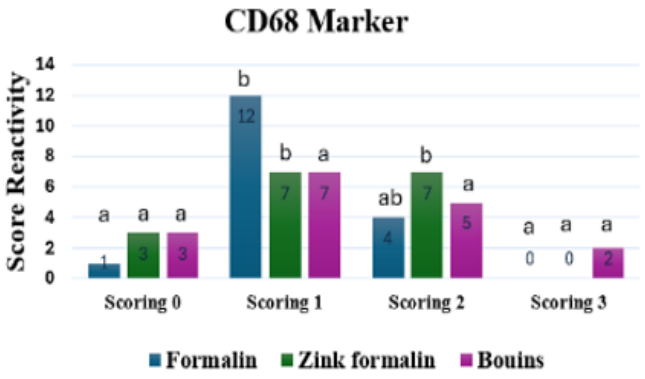

Figure 5: Similar letters specify that there is no significant difference between the fixatives, while different letters indicate that there is a significant variance between the fixatives ($P<0.05$).

Table 5: Pax 8 scoring data

Scoring	0	1	2	3
NBF	0	1	2	3
Zink NBF	0	3	6	8
BF	0	5	8	4

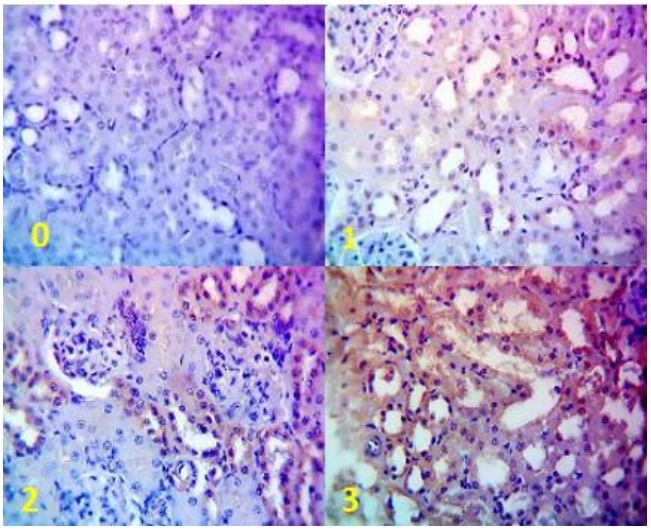

Figure 6: Pax8 marker catalog: Upper left (0) represents score zero, upper right represents score one, and lower left represents score two criteria. The lower right panel represents score three. 400X.

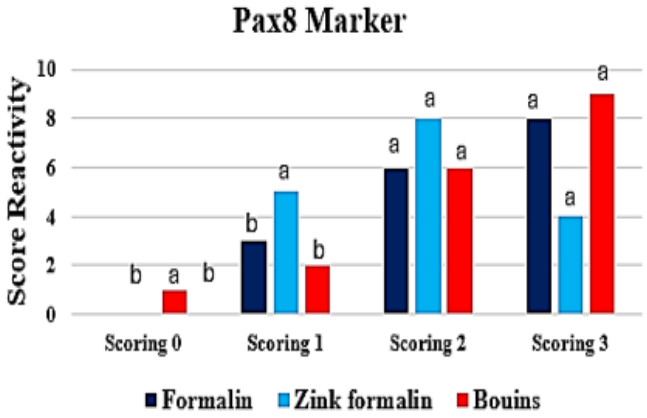

Figure 7: Similar letters specify that there is no significant variance between the fixatives, while different letters indicate that there is a significant difference between the fixatives (P<0.05).

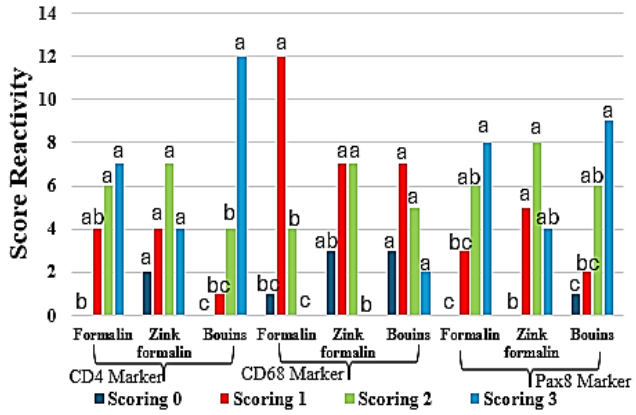

Figure 8: Similar letters indicate that there is no significant variance between scores of the same fixatives, while different letters suggest that there is a significant difference between the scores (P<0.05).

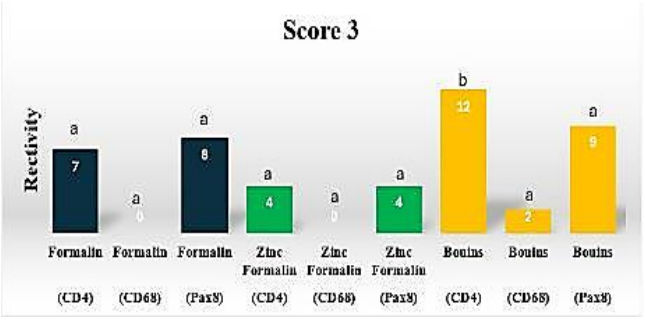

Figure 9: Similar letters specify that there is no significant variance between markers for each fixative, while different letters indicate that there is a significant difference between markers (P<0.05).

Discussion

Various antigenic determinants within normal-specific cell markers in different fixatives have been studied. However, challenges exist in such studies regarding the best fixative of choice to obtain the best antigen retrieval and intact epitopes (29,30). Thus, using inappropriate fixative with standard IHC protocol might affect the best analysis of target cells; when this is applied to our study, these fixatives demonstrate that the antigenic determinants of our target markers CD4, CD68, and Pax8 might affect the accurate analysis by IHC. We designed a scoring system for IHC immunoreactivity to evaluate the positive reaction of each marker for the samples immersed in three different fixatives included in this study. CD4 epitopes were best represented significantly in BF fluid, followed by NBF. However, ZF shows no significant differences compared to BF and F. This result of both F and ZF interaction with the CD4 marker was appropriate for what had been noticed by Mori *et al.* (9).

Since the ZF used in this study is not NBF-free, it masks epitopes from being well-recognized by specific antibodies. As we noticed in our research, CD4 markers were not being detected professionally in all fixative, and this might agree with what had been seen by Holgate *et al.* (31), who mentioned that many monoclonal antibodies have been used today for the detection of lymphocyte surface protein; however, limitations existed as they can be used only in frozen sections. We noticed that CD68 antisemitic determinants were well recognized by both F and ZF; however, BF showed no significance in contrast to the results of the others. Although traditional fixatives did not retain numerous cell surface protein epitopes whereas it could fit others Holgate *et al.* (31), these findings might interpret the significant differences in determining such anti-genetic determinants in both F and ZF than BF; such reason BF was unable to be noticed by CD68. The results of PAX8 IHC of positive PAX8 expression. Our study found that F and BF share the same data when determining Pax8 epitopes. However, ZF revealed no significant variations compared to either F or BF. Also, as mentioned in the result section, score three was statically analyzed for all markers in all included fixatives; this revealed that the intensity and frequency of immunoreactive positive cells showed statistical differences. The CD4 within BF was the best among all fixatives, whereas nothing is shown regarding other markers. Our data reflect no optimal fixative for all antigens; thus, neutral buffered NBF is often a poor fixative for preserving antigen detection by immunohistochemistry (32). According to Huang and Honda (33), about 90% of all known epitopes are conformational. The more complex structure of a protein is comprised of intermittent subsections, which provide difficulties in immunohistochemistry approaches owing to their chemical and structural complexity (33). In brief, no definite fixative was reliable for all antigens (32). This information clarifies what we had concluded regarding the causes behind a variation in detecting some antigenic determinants by some fixative whereas missing in the others in terms of using a different fixative.

Conclusions

In conclusion, NBF showed a good quality result when detecting all epitopes. However, BF fluid was supreme in detecting anti-genetic determinants of CD4 and Pax8 epitopes in score 3. Additionally, ZF could distinguish CD68 and Pax8 but failed to detect CD4; conversely, no significant difference was recorded between B and NBF.

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Conflict of interest

The writers disclosed no conflicts of interest.

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تأثير المثبتات على المحددات الجينية المستضدية لكل من عنقود التمايز الرابع وعنقود التمايز الثامن والستين والجين المقترن الثامن بتقنية الكيمياء النسجية المناعية: دراسة مقارنة بين الفورمالين الدارئ المتعادل والزنك فورمالين ومحلول بونز

أماني عبد الباسط العياش و فراس محمد عبد

فرع الأمراض وأمراض الدواجن، كلية الطب البيطري، جامعة الموصل، الموصل، العراق

الخلاصة

يعد تثبيت العينات مرحلة حاسمة في مجال علم الأمراض، حيث يهدف إلى منع عملية التحلل الذاتي. هدفت دراستنا لتقييم تأثير ثلاثة محاليل مختلفة، وهي محلول الفور مالين المتعادل ومحلول الزنك فورمالين ومحلول بونز، على المضادات الجينية المحددة الخاصة عنقود التمايز الرابع وعنقود التمايز الثامن والستين والجين المقترن الثامن. تم فحص ٥١ مقطع لكل محدد، حيث بلغ العدد الكلي للمقاطع ١٥٣ مقطعا ولتقييم تلك المقاطع تم تصميم معيار لكل قراءة ٠ و ١ و ٢و ٣. أظهرت مقارنة الدرجات الخاصة بعنقود التمايز الرابع بوجود فرق معنوي بين كل من محلول بونز ومحلول الفورمالين المتعادل وكذلك بين البونز والزنك فورمالين، حيث احتل المحلول الدارئ المرتبة الثانية بعد ذلك الزنك فورمالين. ومن المفاجئ أن الزنك فورمالين لم يظهر أي فرق معنوي بين عنقود التمايز الرابع. ومن ناحية أخرى لوحظ أن عنقود التمايز الثامن والستين قد أحرز فرقا معنويا في المعيار ١ لدى كل من المحلول المتعادل وكذلك بالنسبة للزنك فورمالين لكن لا يوجد أي فرق معنوي في محلول بونز. ومن ناحية أخرى أظهرك من المحلول الدارئ ومحلول بونز نتائج جيدة في الجين المقترن الثامن مقارنة بالزنك فورمالين، لكن لم يسجل أي فرق معنوي بينهما. بالرغم من ذلك عند المقارنة بين المعيار الثالث بين جميع المثبتات فان محلول بونز اظهر كفاءة عالية في إيجاد عنقود التمايز الرابع عكس المحلول الدارئ والزنك فورمالين، ومع ذلك، لم يظهر عنقود التمايز الثامن والستين ولا الجين المقترن الثامن أي فرقا. نستنتج من ذلك ان المحلول الدارئ المتعادل كان جيدا في إيجاد جميع الحواتم. ان محلول بونز كان متفوقا في إظهار عنقود التمايز الرابع وعنقود التمايز الثامن والستين في المعيار الثالث. بالإضافة الى ذلك فان الزنك فورمالين كان قادرا على إيجاد عنقود التمايز الثامن والستين والجين المقترن الثامن بينما تعذر في إيجاد عنقود التمايز الرابع وعلى العكس فانه لم يسجل أي فرقا معنويا مع المحلول الدارئ ومحلول بونز.