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GAF vs. Formalin: A turning point in forensic tissue preservation

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ABSTRACT

Formalin's widespread use in tissue fixation for forensic and diagnostic pathology is increasingly challenged by its known carcinogenicity and detrimental effects on biomolecular integrity. This technical note evaluates Glyoxal Acid-Free (GAF) fixative as a superior, non-toxic alternative. We highlight formalin's limitations in terms of occupational hazards and compromised molecular analysis (e.g., DNA degradation for NGS and epitope masking for IHC). Subsequently, we present the advantages of GAF, including excellent morphological preservation, enhanced immunohistochemical performance, and "in press" results about superior preservation of nucleic acids, crucial for advanced molecular techniques. Furthermore, GAF demonstrates remarkable long-term tissue stabilization, supporting its utility for both current and retrospective forensic investigations.

1. Introduction

For over a century, 10 % neutral buffered formalin (NBF) has been the cornerstone of tissue fixation in anatomical and forensic pathology. Its efficacy in preventing autolysis and maintaining gross and microscopic morphology is well-established. However, the continued reliance on formalin presents significant challenges, primarily related to **occupational safety** and its **deleterious impact on biomolecular integrity**, which has significant implications for forensic analysis.

Formaldehyde, the active component of formalin, is a colorless, irritating gas classified as a Group 1 human carcinogen by the International Agency for Research on Cancer (IARC) [1]. Chronic inhalation exposure is linked to various cancers, including nasopharyngeal carcinoma, leukemia and various neurological diseases [2,3]. Its high reactivity and easy assimilation into the human body necessitate stringent safety protocols, extensive ventilation systems, and mandatory worker registration for exposed personnel, imposing considerable operational burdens and costs on forensic and diagnostic laboratories [4]. The European Union's past consideration of a ban, as outlined in EC Regulation n.605/2014 of 05/06/2014, which amended EC Regulation n.1272/2008, although temporarily suspended due to its crucial medical necessity, highlights the global recognition of its potentially hazardous effects and the need of new solutions [5].

Beyond direct health risks, formalin fixation significantly compromises the integrity of nucleic acids and proteins. The formation of methylene bridges through protein cross-linking leads to DNA fragmentation, adduct formation, and nucleotide deamination [6,7]. For forensic investigations increasingly reliant on molecular techniques, this degradation is a major impediment. Next-Generation Sequencing (NGS) and comprehensive genomic profiling (CGP) analyses from formalin-fixed paraffin-embedded (FFPE) tissues often yield degraded DNA, resulting in reduced library complexity, increased sequencing errors, and potential false-negative results [6]. This directly impacts the accuracy and reliability of genetic profiling for identification, familial searching, or establishing predisposition markers in forensic contexts.

Similarly, formalin-induced cross-linking masks epitopes, the specific sites on proteins recognized by antibodies, thereby hindering immunohistochemical (IHC) analysis. While antigen retrieval methods attempt to reverse this masking, their efficacy can be inconsistent, depending on the antigen, antibody, and the duration of fixation [8,9]. Prolonged fixation, common in forensic practice where samples might be stored for extended periods before processing, further exacerbates this epitope masking, leading to decreased or abolished immunoreactivity and potentially compromising the diagnostic or investigative utility of IHC [8–10], although over the years different methods have been proposed to improve results [11–13]. Additionally, numerous alternatives have been studied over the years, but so far, none have demonstrated the necessary characteristics to surpass the use of formalin

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[14].

2. Results and discussion

To address these critical limitations, Glyoxal Acid-Free (GAF) fixative, a new technology patented in 2017 and still under study today, has emerged as a compelling non-toxic alternative. GAF is a glyoxal-based solution specifically formulated without acids, thus avoiding potential pH-related artifacts previously associated with some glyoxal formulations [15,16]. Beyond its non-toxic nature, GAF offers additional intrinsic safety advantages for laboratory personnel. Unlike formaldehyde, which is highly volatile and readily released into the workplace atmosphere, GAF exhibits negligible volatility at room temperature and is devoid of hazard statements (H-phrases) under CLP regulation. As a matter of facts, the reported Henry's law constant of $\leq 3.38 \times 10^{-4}$ Pa m^3/mol at 25°C indicates that glyoxal is essentially non-volatile in its aqueous phase [17], substantially reducing the risk of inhalation exposure during routine handling, sample processing, and storage operations. The low vapor pressure of GAF also minimizes the need for extensive ventilation systems and reduces the potential for inadvertent exposure, particularly during extended laboratory procedures common in forensic investigations. However, citing Shakespeare in the Merchant of Venice, "*all that glitters is not gold*", cause contact between glyoxal and skin is proved to cause dermatitis and allergic reactions with a very strong skin sensitizing potential, underlining the need of attention when handled[18].

2.1. Morphological and molecular preservation

Studies have consistently demonstrated that GAF provides excellent tissue fixation, yielding morphological preservation comparable to, and in some aspects superior to, NBF [5,19]. This includes maintaining the original consistency and color, and clear visualization of cellular detail and tissue architecture, essential for accurate forensic histopathological examination. Historically, some promising alternatives to formalin, such as NoTox, have failed to meet the required performance standards. This is because, despite their low or absent toxicity and good microscopic performance, they resulted in a friable anatomical block that was difficult to work with in micrometric sections [20].

Regarding the results at the genomic level, GAF seems to improve the preservation of nucleic acids. DNA extracted from GAF-fixed paraffin-embedded (GFPE) samples exhibits markedly higher yield and superior integrity (as measured by DNA integrity number, DIN) compared to NBF-fixed samples, demonstrating less fragmentation and better quality for downstream molecular applications like PCR and NGS [6,19,21]. This is vital for forensic DNA profiling where sample quality is often a limiting factor.

Preliminary data from a "in-publish" study [22] revealed promising results for GAF as a fixative. DNA yields from GAF-fixed biopsy of animal tissues were significantly higher compared to NBF-fixed tissues ($P < 0.05$), with more efficient amplification of selected genes like TP53 and COX1. While not statistically significant, necropsy GAF-fixed samples also exhibited a tendency towards higher DNA concentrations. RNA extraction from canine tumor biopsies consistently showed higher yields in GAF-fixed samples compared to NBF, although this difference was not statistically significant. Furthermore, RNAscope analysis confirmed positive detection of c-KIT mRNA in GAF-fixed samples, comparable to NBF-fixed controls, indicating effective RNA preservation. These findings ulteriorly confirm that GAF offers improved preservation and accessibility of nucleic acids for downstream molecular analyses, opening to more in-depth research in forensic settings also simplifying the use of "omic" investigation.

2.2. Immunohistochemistry analysis

GAF maintains the antigenicity of a broad panel of diagnostic markers, demonstrating high comparability in IHC results with NBF, for example, KI67, ER, PGR, and HER-2 in a clinical setting [5]. The reduced cross-linking inherent to glyoxal-based fixation means less epitope masking, potentially reducing the need for aggressive antigen retrieval techniques and improving the consistency and reliability of IHC staining, which is highly beneficial for the diverse and often challenging samples encountered in forensic pathology as wound-vitality estimation [23,24]. These results are consistent with observations made regarding unbuffered Glyoxal, indirectly confirming the excellent premises of GAF, which retains its positive aspects [25,26].

As an example, the dataset from the aforementioned Riska et al. article[5] can be fully consulted, including in a blinded fashion, on the Addax Biosciences website (https://addax.crs4.it/datasets/1), where are also provided high-precision scans of the samples used, stained with H&E and various IHCs.

2.3. Long-term stability

Preliminary data from long-term studies indicate that GFPE tissues maintain their morphological and molecular integrity effectively even after 10 years, comparable to or exceeding the performance of FFPE tissues over similar durations [21]. Similar results have been observed in the forementioned "in press" article, with particularly promising results on DNA-stability index[22]. Obviously, other studies are needed to confirm these data cause long-term stability is paramount for forensic evidence, ensuring compliance with legal retention limits and enabling reliable retrospective analyses years after initial collection, potentially representing a "turning point" for its use in the field.

3. Conclusion

The persistent challenges posed by formalin, specifically its human carcinogenicity and adverse impact on biomolecular integrity, necessitate a shift towards safer and more effective tissue fixation methods. Glyoxal Acid-Free (GAF) fixative would represent a robust technical solution for forensic and diagnostic laboratories. If confirmed by further studies, its non-toxic nature, coupled with superior morphological preservation, enhanced immunohistochemical performance, and, crucially, high-fidelity preservation of nucleic acids for advanced molecular profiling, makes GAF an ideal alternative. The long-term stability demonstrated by the analysis of some of the first samples further solidifies GAF's potential as the preferred fixative for future forensic pathology practices, safeguarding both laboratory personnel and the integrity of invaluable evidence. The limitations of neutral-buffered formalin (NBF) have been incontrovertibly and definitively demonstrated, particularly concerning new molecular diagnostic technologies. This increasingly urgent need mandates a transition to a new technology that ensures superior results and keeps pace with current advancements and GAF can represent a valid alternative. Naturally, we're still in an embryonic phase of the technology, and we lack studies that can only be carried out with a general increase in interest and a subsequent rise in the use of this fixative.

CRediT authorship contribution statement

Matteo Perilli: Writing – review & editing, Writing – original draft, Supervision, Data curation. **Massimo Montisci:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Formal analysis, Data curation, Conceptualization. **Rossana Cecchi:** Supervision. **Michela Gastaldi:** Supervision, Data curation, Conceptualization. **Toshikazu Kondo:** Validation, Supervision.

Declaration of Competing Interest

The Authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

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