

RESEARCH ARTICLE

HONEY AS FIXATIVE AND SAFER SUBSTITUTE FOR FORMALIN IN HISTOLOGY

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ABSTRACT: Formalin is a popular fixative and biohazard in histology and pathology laboratories. The objectives were to investigate the physiochemical properties and effect of different concentrations of honey as fixative on gross morphology and histology of tissues. Honey's glucose concentration and pH were measured. Five organs from goat were gotten in pairs and small tissues cut before being grouped into A and B. Group A were fixed in 20%, 50%, 70%, 90%, 100% honey concentrations while group B were fixed in 10% buffered formal saline for 48 hours-6 months. The cut tissues were processed by paraffin wax-embedding method and stained with haematoxylin and eosin. Glucose concentration was 284mmol/l and pH was 4.0. Macroscopically, tissues fixed in 20% and 50% honey showed putrefaction changes after 72 hours and were statistically significant ($p=0.004$). Tissues preserved in 70%, 90% and 100% honey were properly fixed for up to 6 months and beyond. Minor differences in nuclear and cytoplasmic staining ($p=0.391$), intensity and clarity of histological details ($p=0.252$) among the honey-fixed group were not statistically significant. In conclusion, the 70-100% honeys are suitable for long term gross preservation while 20-50% concentrations give excellent tissue staining characteristics. Thus, honey is a safer substitute for formalin.

KEY-WORDS: Formalin, honey, fixation, histology, substitute

INTRODUCTION:

Fixation is an important step in tissue processing¹ and formalin is one of the routine fixatives used in histology and pathology laboratories^{2,3}. Formalin is also a key preservative in embalming fluids^{4,5} and in museum studies⁶. However, formalin poses great health and safety risk to humans^{4,5,7,8}. Formalin causes cancers, irritation of skin, mucous membranes of eyes, respiratory tract and gastrointestinal tracts, neurotoxicity, reproductive anomalies and developmental defects^{4,5,8}.

Due to these toxicities, honey was used as a substitute for formalin. Honey is a natural and non-hazardous fluid used as fixative since ancient times^{7,9,10}. Honey is produced by the stinging bee *Apis mellifera* or by the stingless *Melipona* and *Hypotrigona* species¹¹. Honey's ability to fix tissues is as a result of its antibacterial, anti-autolytic, antimicrobial, antioxidant, and tissue hardening properties^{7,10}. Different authors have established the use of honey as fixative in

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histology and cytology^{1,7,9,10,12}. But there is paucity of data relating the different concentrations to gross long term fixation (preservation) and histological morphology. The rationale of this study was to evaluate the fixing abilities of different concentrations of honey (20%, 50%, 70%, 90%, 100%) on long term preservation meant for embalmment and museum studies and for microscopy using different tissues of goat. This work has shown that honey is a safer and good fixative which can be substituted for formalin in histology.

SUBJECTS AND METHODS:

Experimental animal and organs

Fresh tissues of heart, intestine, lungs, kidneys, and brain of a goat were purchased at Atakpa Abattoir, Calabar, Cross River State, Nigeria.

Source of honey/authentication of honey

The honey was purchased from a local dealer in Taraba State, Nigeria. The authentication of the honey was done using the match light test method as stated by¹³.

pH of honey: The pH of the honey was determined using a pH meter (pH 211-02, Hanna Instruments, Romania). This was done following the manufacturer's instruction after diluting 10g honey in 75ml distilled water.

Glucose content: This was done using the glucose-oxidase colorimetric method of Randox Laboratories, United Kingdom based on the principle of glucose oxidase-peroxidase-aminophenazone. Absorbance was read at 540nm using a colorimeter and glucose concentration calculated in mmol/l. The value obtained was multiplied by the dilution factor of 10

Gross fixation of tissues

The tissues of the goat were bought in pairs and immediately photographed before drying. For

honey fixation, the tissues were washed in 20% honey before they were fixed in different concentrations of 20%, 50%, 70%, 90% and 100% in large containers. The tissues were checked after durations of 48 hours, 1 week, 2 weeks, 1 month, 3 months, and 6 months. The formalin fixed tissues were washed in 10% formalin to remove blood and fixed in 10% buffered formalin for the same duration of the honey-fixed counterparts.

Fixation for histological tissue processing

Each of the 5 tissues from the goat was assigned into two (2) groups, A and B. The group A tissues were fixed in different concentrations of honey (20%, 50%, 70%, 90% and 100% respectively) for 48 hours while the group B tissues were fixed in 10% buffered formalin for 48 hours. After 48 hours, all tissues were cut into tiny bits for tissue processing.

Tissue processing

All tissues were processed with the paraffin wax embedding method before they were sectioned at 4µm with a rotary microtome and sections picked onto albumenized glass slides for haematoxylin and eosin staining¹³. Sections were stained in Cole's haematoxylin for 10 minutes, counterstained in 1% eosin for 30 seconds, dehydrated, cleared and mounted in DPX¹³. The sections were viewed and photomicrographs were taken using a Leica DM500 microscope.

Statistical analysis

The Statistical Package for Social Sciences (SPSS) version 20.0, IBM Incorporated USA was used to analyze the results. Student T- test was used to calculate the associations between variables. Probability level of $p \leq 0.05$ was statistically significant.

RESULTS:

Physiochemical properties: The honey used in the study was good with a deep amber colour, pH

was 4.0 at temperature of 27°C and glucose content was 284mmol/l as shown in Table 1.

Gross fixation study

Table 2 shows the effect of different concentrations of honey and 10% buffered formalin on gross fixation. Tissues fixed in 20% and 50% honey gave poor fixation with total score of 3(14.3%) after 72 hours and was statistically significant ($p=0.004$). While tissues fixed in 70%, 90% and 100% honey gave good preservation for up to 6 months with total score of 21 (100%) similar to the formalin-fixed counterparts.

Microscopic study

Table 3 shows the staining characteristics of tissues fixed in different concentrations of honey. The 100%, 90% and 70% concentrations gave good intense and clear nuclear and cytoplasmic staining with moderate preservation of tissue morphology with total scores of 57(95%), 57(95%) and 56(93.3%) respectively. The 50% and 20% gave good intense and clear nuclear and cytoplasmic staining with good preservation of tissue morphology with total scores of 59(98.3%) each. The differences in staining characteristics were not statistically significant when compared with the formalin-fixed counterparts for 70% ($p=0.252$) and for 20%, 50%, 90%, 100% ($p=0.391$) respectively.

Photomicrographs

The photomicrographs are shown in Figure 1 and 2. Figure 1 shows sections of the small intestine fixed with 10% formalin (Fig.1A) and the different honey concentrations (Fig1B-D) with prominent layers comprising of the serosa (SE), muscular (M), submucosa (SM) and mucosa layers (MUC) in Figure 1A-D. Figure 2 shows sections of lungs with prominent alveoli spaces (ALS) and pneumocytes (PN) fixed in 10% formalin in Figure 2A and 50% honey in Figure 2B. The well stained cardiac muscles (CM) and their nuclei (N)

in Figure 2C were fixed in 10% formalin and in Figure 2D fixed in 50% honey.

Table 1: Physiochemical properties of honey

Physiochemical properties	Colour	pH	Temperature	Glucose content
	Deep amber	4.0	27°C	284mmol/l

Table 2: Scores for effect of formalin and different honey concentrations on gross fixation

Duration	10% formalin	20% honey	50% honey	70% honey	90% honey	100% honey
48 hours	3	3	3	3	3	3
72 hours	3	0	0	3	3	3
1 week	3	0	0	3	3	3
2 weeks	3	0	0	3	3	3
1 month	3	0	0	3	3	3
3 month	3	0	0	3	3	3
>6 months	3	0	0	3	3	3
Total	21(100%)	3(14.3%)	3(14.3%)	21(100%)	21(100%)	21(100%)
		t=5.000 p=0.004	t=5.000 p=0.004			

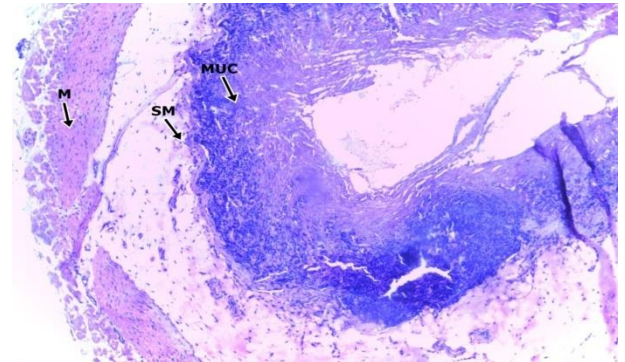
Key: 0-No fixation (autolysis and putrefaction present), 1-Poor fixation, 2-Moderate fixation, 3-Good fixation (autolysis and putrefaction absent).

Table 3: Scores for staining characteristics of formalin and different honey concentrations

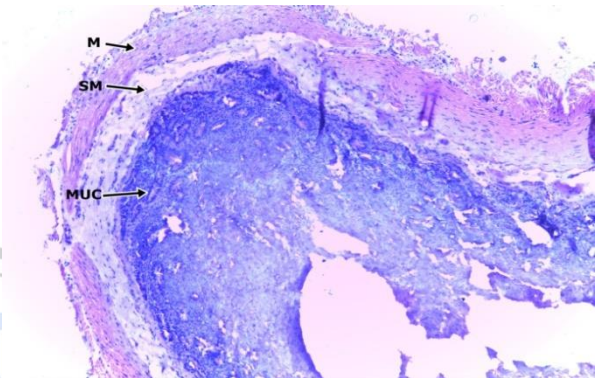
Staining characteristics	10% formalin	20% honey	50% honey	70% honey	90% honey	100% honey
Nucleus	15	15	15	15	15	15
Cytoplasm	15	15	15	14	15	15
Clarity	15	15	15	14	14	15
Morphology	15	14	14	13	13	12
Total	60(100%)	59(98.3%)	59(98.3%)	56(93.3%)	57(95.0%)	57(95.0%)
t values		1.000	1.000	1.414	1.000	1.000
p values		0.391	0.391	0.252	0.391	0.391

Keys: 1-Poor, 2-Moderate, 3-Good nuclear/cytoplasmic staining, intensity and clarity.

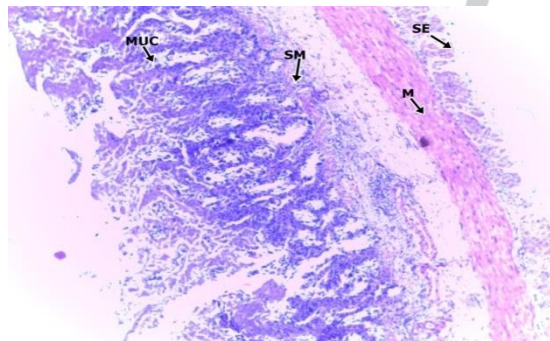
(Note: Sum of scores were derived as follows: 15=(3x5), 14=(3x4+2), 13=(3x4+1) and 12=(3x3+2+1) The number of tissues were 5.)



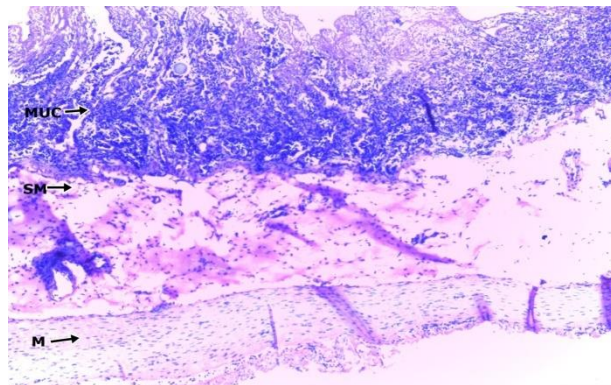
C: 70% honey



D: 100% honey

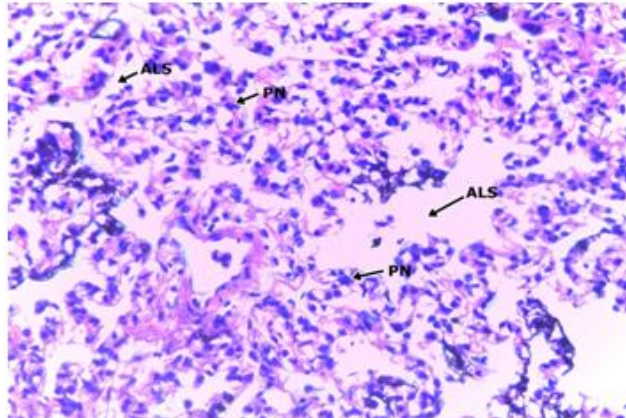


A: 10% formalin

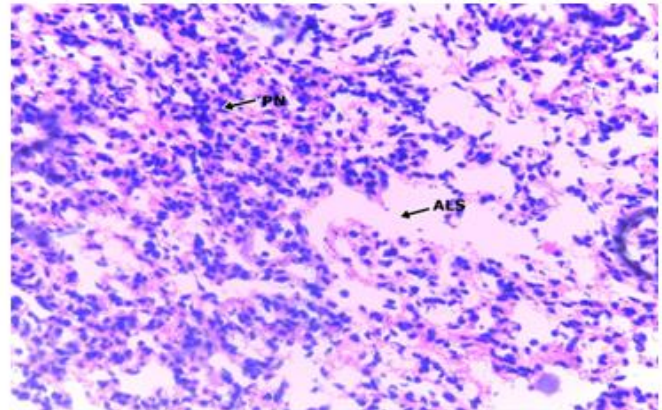


B: 20% honey

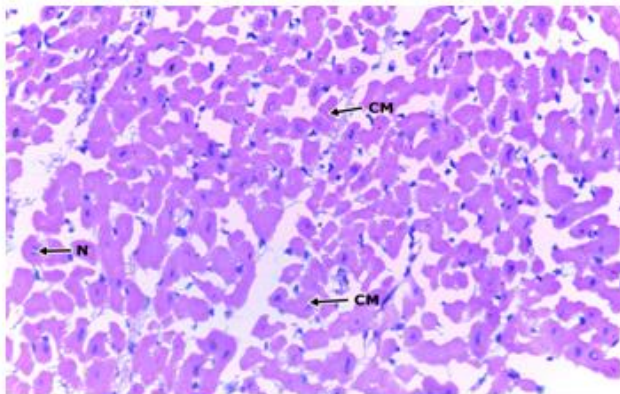
Fig.1: Small intestine sections fixed in formalin and different concentrations of honey. There is prominent serosa (SE), muscular (M), submucosa (SM) and mucosa layers (MUC) in A, B, C and D. H&E X100



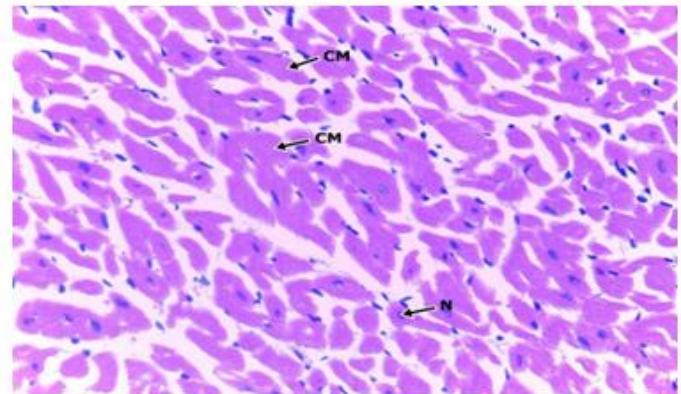
A: 10% Formalin



B: 50% honey



C: 10% Formalin



D: 50% honey

Figure 2: Lungs and cardiac muscle sections fixed in 10% formalin and 50% honey. There are prominent alveoli spaces(ALS) and pneumocytes (PN) in lungs in A and B. The cardiac muscles (CM) and their nuclei (N) in C and D are well stained. H&E x400.

DISCUSSION:

Formalin fumes make the laboratory unsafe for the workers and students when inhaled but its use as fixative and embalming fluid cannot be ignored because there are no safer and better alternatives in our environment ⁵. Fixation stabilizes tissue proteins to preserve the tissues in a life-like manner after they have been removed from the body ¹. As such, a fixative must properly maintain the constituents of the tissues to allow for good staining and microscopy.

The fixing properties of honey can be attributed to several factors such as acidic pH, low moisture and high sugar content. The acidic pH of 4.0 in this study ensured that most micro-organisms were killed allowing a sterile environment for the honey and tissues to remain for a long time ¹⁵. The acidic pH is derived from organic acids such as gluconic acid, acetic, lactic, citric, formic and fumaric acids ¹⁶. This acidic pH falls within the ranges reported by ¹¹ in Enugu, ¹³ in Adamawa and ¹⁷ in Kwara states of Nigeria.

The results obtained showed that for long term gross fixation and for histological staining of the

tissues, higher concentrations of 70%, 90% and 100% gave good preservation beyond 48 hours. This is because honey is a hygroscopic (low moisture) substance because of its high sugar content. Also micro-organisms do not survive long in a low moisture environment. Thus, at these concentrations the water content is lesser and did not over dilute the sugar content of the honey. The result of the gross morphology can be compared with result of ¹. This shows that honey at high concentrations are suitable for museum studies and for embalment purposes. This further justifies the use of honey for embalment since ancient times ^{4,6}.

The ability of honey to fix tissues has been attributed to its antibacterial, antimicrobial and antioxidant properties ¹⁰. The acidic pH and low water content ensure that bacterial and other micro-organisms do not survive in honey ¹⁶. The antioxidants present in honey are enzymes such as catalase, glucose oxidase and peroxidase. Other non-enzymatic substances are ascorbic acid, phenolic acids, flavonoids and Maillard reaction products^{11,16}. These antioxidants preserve the tissues by preventing lipid peroxidation ¹⁶.

Two mechanisms of fixation by honey have been postulated. First, fixation occurs due to the formation of hydroxymethylfurfural (HMF) which is an intermediate product formed in the Maillard's reaction. The HMF then forms a cross link with the tissues through the di-Schiff base reaction¹². Second, Patil *et al.*¹ had stated that fixation occurs when the sugars, largely fructose, are broken down at acidic pH to form aldehydes which in turn forms cross-links with the tissue proteins. This protein cross-linking is similar to the reaction that occurs with the use of formalin ².

The use of honey in this study has good results similar to reports by^{1,7,9,10,12,18} in different concentrations ranging from 1%-100% honey.

CONCLUSIONS:

This study has shown that higher concentrations of 70-100% honey are suitable for long term gross preservation of tissues and organs while lower concentrations of 20-50% give excellent tissue staining characteristics. Thus, honey is a good alternative to formalin because it is natural, health and environment safer, non-carcinogenic, non-irritating and non-volatile preservative and fixative.

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