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SHORT COMMUNICATION

Evaluating Three Histochemical Stains (Solochrome Azurine Stain (Asa), Walton Stain, and Modified Hematoxylin) used in Tissue Aluminium Detection.

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SUMMARY

Aluminium in recent times has been considered a possible risk factor in some diseases in humans, animals and plants, and exposure to aluminium may pose a health hazard. Studies have pointed to the fact that increasing acidification of the environment has made aluminium more bio-available and therefore, able to cause disturbances in the function of human and animal organisms. More importantly also, is the use of aluminium as based adjuvants in human vaccinations, and its fate being unclear. Our study aimed to evaluate histochemical stains currently used to detect Al in tissue samples for their sensitivity using agar blocks as a preliminary study to validate the Walton histological stain use in detecting aluminium toxicity in fish. Visual estimation (colour change and staining intensity) of aluminium-stained sections using the Solochrome Azurine stain (ASA), Walton stain, and the modified haematoxylin were carried out. All three stains indicated the presence or absence of aluminium through colour changes, but the ASA gave more distinct dose- response intensity in staining.

Keywords: Aluminium toxicity, Walton stain, Solochrome azurine, modified haematoxylin.

INTRODUCTION

Aluminium is an abundant metal comprising about 8% of the earth's crust. However, its concentration in water, foodstuffs, and biological media is usually low (Elinder and Sjogren, 1986). Increased levels of bio-available forms in the environment are because of acidification which mobilizes Al from the earth crust reserves into surface waters (Cronan and Schoefield, 1979; Dickson, 1980; Yamamoto 2019).) contributing to the mortality of fish at low pH (Baker and Schofield, 1982; Muniz and Leivestad, 1980; Poleo, 1997) and probable cause of the fish mortality linked to respiratory and acid base disturbances (Peuranen et al. 2003). Likewise, vegetation and organisms living in acidified areas may show elevated concentrations of aluminium.

There are several routes of exposure to Al. Exposure to aluminium may pose an occupational hazard; in humans, symptoms of obstructive lung disease have been reported from workers at aluminium production plants (Elinder and Sjogren, 1990; Kongerud and Søyseth, 2014). There have been reports of pulmonary fibrosis originating from deposit of stamped aluminium powder and dust in the lungs,

as well as long term exposure to aluminium by inhalation giving rise to its accumulation even in the body (Elinder et al., 1991; Cai, 2007). Aluminium is also considered a neurotoxin that occurs in human brain (Xu et al, 1992; Yoshida et al., 1996; Andra- si et al., 2005; Walton, 2006) and is linked to Alzheimer's disease (Exley, 2014; Exley and Clarkson, 2020).

The ingestion of aluminium additives is the major route of aluminium exposure by the public other than occupational hazards and chronic aluminium antacid uses (Krewski, 2007; WHO, 2003). Aluminium sulphate (alum) is also added to some water for bottling as drinking water and many urban drinking water supplies to clarify them. The toxic effects of Al ranges from reproductive toxicity (Muoro et al, 2018) to bone abnormalities (Klein, 2019) and neurologic disorders (Colomina and Peris-Sampedro, 2017).

Conventional analytical techniques such as atomic absorption spectrometry (AAS) or inductively coupled plasma (ICP) spectrometry used to detect Al in tissue samples cannot be performed easily hence the use of histochemical stains such as Solochrome Azurine (Kaye et al., 1990), modified haematoxylin (Havas, 1986) and the Walton stain (Walton, 2007), which are frequently employed to detect and assess the distribution of Al in tissues.

Solochrome Azurine has been described in many reports as being more sensitive than Aluminon (Kaye, et al., 1990, Hodsman and Steer, 1992; Fernandez-Martin et al.; 1996, Ruster et al., 2002) which is reported a negative or weakly positive results in Al detection leading the widespread and erroneous belief that Al exposure and toxicity are absent (Cannata-Andia, 2001). Although Solochrome Azurine has been used widely as an indirect marker of bone aluminium content (Kaye et al, 1990; Fernández-Martín et al., 1997).

Haematoxylin was first described as a useful biological stain in 1865 and rapidly became one of the most extensively used stains in the study of living organisms (Conn, 1929). Havis (1986) reported that haematoxylin stain can be used to locate sites of AI binding in freshwater plants and animals and (Exley, 1996) reported neuropathology of the brain identified with modified haematoxylin stain in aluminium-exposed fish.

The Walton histological method is reported to have a straightforward mechanism for staining aluminium, (Karlik et al., 1980; Martin, 1988). The Walton method appears to be as sensitive in bone as in soft tissues and it yields highly reproducible results (Walton et al., 2007).

As a preliminary study to evaluate, compare and validate the use of these histochemical reagents in aluminium fish toxicity, we incorporated different concentrations of Al in plain agar cut into blocks and employed published histological methods or techniques for staining Al using these reagents to establish a rapid and reliable screening system.

Samples were stained by direct immersion into the staining solution. The results of the different staining techniques were compared through visual examination.

MATERIALS AND METHODS

A 0.34g plain agar in 20ml distilled water was autoclaved for 15mins at $121^{\circ C}$ in 50ml Smart's bottles. A 10mg/ml stock solution of Aluminium chloride ($AlCl_3$) was prepared to make a 200µg/ml, 400µg/ml, 1000µg/ml solution which were immediately added into the bottles after autoclaving and was gently mixed. A concentration with no $AlCl_3$ served as the control.

After autoclaving, the agar was poured into petri dishes which received a uniform 20ml plain agar broth containing 0, 200, 400, 600, 1000µg/ml of $AlCl_3$ which upon cooling were cut into blocks of agar which were stained in triplicates, the experiment was repeated thrice.

Staining techniques

Cellulose fibres of 2 cm discs were processed for the three different staining techniques employed. For the Walton stain, the agar discs were stained overnight in chromogallocyanin after which they were washed in distilled water and stained in 0.5% phloxine B for three minutes. The discs were then washed to remove excess stain and placed in 5 % phosphotungstic acid for one minute, and then washed before being differentiated in 80% ethanol for two minutes. It is then stained in 0.05% fast green FCF for three minutes and washed in 1% glacial acetic acid for one minute. The sections were later dehydrated rapidly in two changes of 95% and 100% ethanol.

A fresh solution containing 2g haematoxylin (anhydrous Merck®) and 0.20g sodium iodate dissolved in 1 L distilled water was used as the modified haematoxylin stain. The discs were put in the stain for 30minutes, after which they were rinsed with distilled water.

The staining procedure for the Solochrome azurine stain involved dissolving a 0.2g of Solochrome azurine (syn Pure blue B Standard Fluka ®) in 100ml distilled water. The discs were placed for 20mins in the stain, washed in distilled water and lightly counterstained in 0.5% aqueous neutral red for 5 minutes.

Visual estimation and comparison of the sections that had been incubated with aluminium solutions and those of the control were made. The staining intensity difference between the stains used were also observed and graded. Three concentrations were picked for final analysis (0µg, 400 µg, and 1000 µg).

RESULTS

Quantitative staining results

The three conventional histochemical stains consistently demonstrated presence of Aluminium chloride. The gelatine sections which contained Al revealed specific colour changes relative on the stains used and comparison made between the sections that had been incubated with aluminium solutions and those that did not contain aluminium solution. Using the Walton procedure for staining Al, the presence of Al was detected as a deep purple/ magenta coloration using the Walton stain (Fig 1 below). The control sections which had no Al, stained brown as they appeared to have picked some of the colour of the stain, although Al was not present.
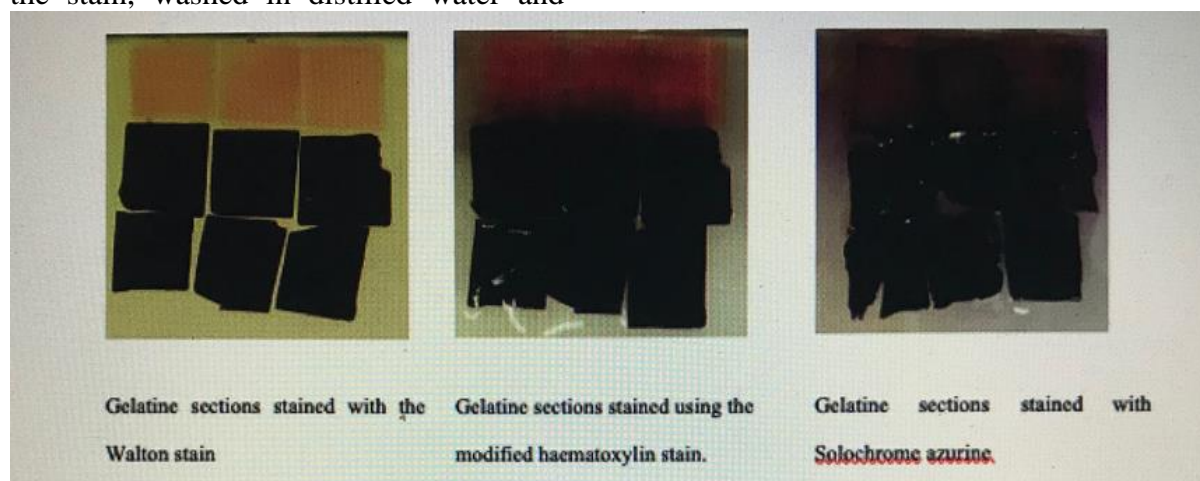

Fig 1 Gelatin sections showing stains used Walton, Haematoxylin and Solochrome

The Walton stain did not stain in a dose-response fashion with the concentrations used, as the intensity of staining did not increase with increasing concentration of Al. Nonetheless, a magenta coloration

change was detected in sections containing aluminium.

The modified haematoxylin stained in a similar fashion to the Walton stain. It appeared as a deep purple coloration (Fig 2) in sections containing Al and stained as dark brown for the control, while The ASA stain shown in Fig 2 stained light to dark purple in a dose response fashion.
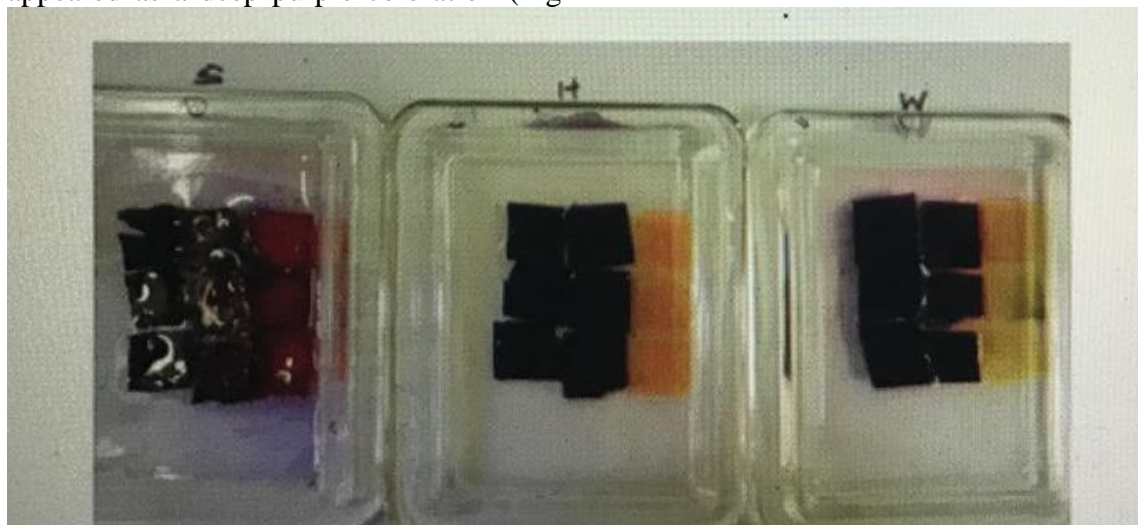

Fig 2 Showing all three stains used, S- Solochrome azurine; H - modified haematoxylin, W- Walton stain 200µg/ml. 400 µg/ml, 1000 µg/ml AlCl₃ solution

The Walton and haematoxylin stains gave marginally more intense staining (always scoring 2) than solochrome azurine stains (scoring 1–2, Table 1).

Table 1 Histochemical stains demonstrating aluminium-containing particles embedded in gelatine sections

Stain type	Control	AlCl₃
Walton stain	0	2
Modified haematoxylin	0	2
Solochrome azurine	0	1-2

Scoring system ranges from no visible stain (0) to clear specific staining (2).

The degree of stain was estimated and scored on a scale of 0–2, where a score of 0 = particles not stained; 1 = particles stained more compared to the gelatine control; 2 = a slightly heavier stain on the particles than 1.

DISCUSSION

Currently there is not a widely accepted and cost-effective method to detect aluminium in histological tissue samples in laboratory settings. When metals such as Al binds, it involves a hard Lewis acid binding to oxygen donors in phosphate and carboxyl groups of protein (Karlik, 1980; Martin, 1988). Al is known to bind covalently to tissue components to form a complex with the carboxylate group of the phoxine dye, an eosin homologue. Aluminium thus acts as a mordant, forming a stable intracellular magenta-coloured aluminium lake (Ghorpade, 1995), hence, the Walton stain uncovers aluminium bound to organic molecules in cellular and sub-cellular structures (Walton, 2004).

In plant or animal tissue, Al is known to act as a mordant and it binds hematein (oxidized haematoxylin) to indicate sites of AI binding, when the haematoxylin stain is used (Havas, 1986). Haematoxylin, colour tissue sections which allow for viewing of the presence or prevalence of cell types, structures or even microorganisms such as bacteria (Anderson and Rolls, 2021). Giaveno and Filho 2000 investigated the use of hematoxylin staining to detect Al-tolerant plants at the seedling stage, a method that helped detect aluminium toxicity which is the major factor limiting plant growth in acidic soils. This reaction occurs by the oxidation (in the presence of $NaIO_3$) of hematoxylin to hematyn, which in the presence of aluminium produces nucleic acid coloration (Cançado, 1997; Myers, 2021).

Denton and Oughton, 1993 proposed the utilization of the ASA stain in assessing the Al content and its distribution in organisms exposed to acidified waters. Ellis et al., 1988 reported that solochrome azurine staining indicates a more extensive distribution of aluminium in both compact and cancellous bone than is often apparent with aluminon, a commercially prepared stain used in Al detection. Histochemical stains, aimed at detecting aluminium in tissue sections, rely on chelation of the metal ion by a chromogen which yields a change in colour or fluorescence that is detectable by microscopy. This then localises aluminium at the cellular level (Powell, 2002).

In this study, the gelatine sections impregnated with Al was readily detected by the stains evidenced by colour change. The Walton and modified haematoxylin stains detected the presence of Al in a none-dose response fashion. This perhaps was due to its sensitivity at identifying lower concentrations of Al than the Solochrome azurine stain, which displayed a dose- response fashion to the presence of Al. This lends further credence to the report of the Walton stain as a better stain

for Al detection and its use as a histochemical Stain (Walton, 2004). The presence of Al using Walton and modified haematoxylin staining could therefore be a more practical, simple, reproducible, sensitive, and cost-effective histological technique to detecting or predict presence of Al in samples.

The Solochrome azurine graded response to Al, may serve as a valuable diagnostic tool useful for detecting rated Al concentrations in samples and could be explored for this purpose especially as Aluminium strips for detecting Al contents in liquid samples.

CONCLUSION

We recommend that the Walton or haematoxylin staining methods should either be used to replace the ASA especially when detecting lower limits of Aluminium is anticipated.

One general problem with the Solochrome Azurine reagent is that it is becoming increasingly difficult to obtain. The use of ASA methods results in staining reagents precipitating shortly after they are prepared, requiring that tissue sections remain immersed overnight amongst the precipitates, which may lead to artefactual precipitates.

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