Original Research

Determination of Sex by Dental Pulp Tissue - A Single Blind Study

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ABSTRACT

Background -Determination of sex in cases of natural disaster, military conflict, terrorism and mass transportation accidents becomes essential to identify the deceased person. Various tools are used to ascertain the sex determination in forensic medicine. Aim of the study: In the present study, we intend to evaluate the presence of Barr body in the histologically processed human dental pulp tissue taken from the freshly extracted teeth.

Study design:- It was a single blind in-vitro study consisting of 40 healthy teeth extracted from both male and female subjects in the age range of 18-45yrs. The samples were processed and evaluated by the principal investigator and the results were correlated with that of the evaluator.

Materials and methods:- The freshly extracted teeth stored in 10 % formalin were split and pulp tissue was taken out, processed and subjected to acridine orange staining as described by Von Bertalanfy et al. Stained sections were examined under fluorescent microscope and the percentage of Barr body positive cells was determined. Results: The data was statistically evaluated using chi-square test, the sensitivity came out to be 96% and specificity as 87%. The percentage of Barr body positive cells in male samples ranged from 0-2% while in female sample ranged from 19 to 48%.

Conclusion:- The presence of Barr bodies in dental pulp tissue proved to be a useful tool in the determination of sex in forensic odontology. This study can further be extended in the evaluation of human identification in natural calamities and mass disasters, when the body is mutilated beyond the scope of identification.

Key words:-Forensic odontology, Barr bodies, human dental pulp, acridine orange, fluorescent microscopy.

INTRODUCTION

In modern society, where the crime graph is high, as well as mass casualties and large scale disasters have caused loss of human resources. In such cases, it becomes a pre-requisite for the identification of the deceased for the law as well as for resolving the apprehension experienced by the grieved family members.[1]

Today we consider forensic odontology to be a specialised and reliable method of identification of the deceased, particularly in multiple fatality incidents where the bodies are mutilated, burned or decomposed beyond the scope of identification.[2]

Determination of sex is one of the most important steps for the identification of the deceased individuals. The process becomes easy in cases where the bodies are well preserved, but it becomes a challenge for the forensic inspector when only few parts of the body, such as teeth or bone pieces are available for evaluation.[3,4]

Teeth are one of the hardest structures in the human body and tooth pulp being embedded in a hard tissue casting can resist the detrimental effects of impact, heat and trauma. So, it can be considered as a valuable tissue for the identification of gender.[5,6]

One of the method of identification of sex from dental pulp tissue is the detection of X-chromatin. The Barr body or the X-chromatin, is about 1 micrometer in diameter and can be seen as a basophilic, condensed structure located near the inner surface of the nuclear membrane in the interphase of female somatic cells.[7]

Many studies have been done to determine the presence of Barr body using H & E stain for histological sections of the dental pulp tissue. However, the application of a chromatin specific dye such as Acridine orange for the same purpose has not been explored. With this view in mind, we intend to evaluate the presence of Barr body within the fibroblasts of the human dental pulp tissue using acridine orange fluorescent dye.

Materials and methods

Methodology or study design

It was an in-vitro single blind study in which 40 freshly extracted teeth samples were included. The teeth were obtained from both male and female cases who visited the OPD of the oral surgery department.

The inclusion criteria for the selection of teeth in this study were:-

1. Healthy teeth extracted from both male and female patients between the age range of 18 -45 yrs.
2. Premolars extracted for orthodontic purpose
3. Periodontally compromised teeth
4. Prophylactically extracted impacted third molars

Exclusion criteria:-

1. Carious teeth.

The teeth extracted by the oral surgeon were placed in separate bottles which were labelled from 1 to 40 (figure-1).

The details of the sample were recorded by the oral surgeon and later the samples with the details were handed over to the evaluator. The evaluator handed over samples to the principal investigator for further processing and evaluation of tissue. Hence, utmost care was taken to keep the study single blinded.

The results obtained by the principal investigator were correlated with that of the evaluator. The data was statistically evaluated using a chi-square test and the sensitivity and specificity was obtained.

Procedure

The teeth collected were washed with sterile water to remove

the residual blood, and stored in the container filled with 10%formalin.(figure 1&2)

On collection of the sample, the procedure followed by the principal investigator was as follows:-

In order to extirpate the pulp tissue, the tooth was longitudinally sectioned with the help of the flexible diamond disc attached to a micromotor running at the lowest speed. This was done to create a wedge in the enamel. The tooth was then split by placing the chisel (at the junction of the crown and root) into the wedge, and then splitting it using a mallet(figure 3).

The pulp tissue was separated out of the pulp cavity with the help of the needle and forceps and transferred to a conical tube containing 10%formalin, processed and formalin fixed paraffin blocks were made (figure 4&5). Serial sections with a thickness of 5microns were taken and 2 different slides were prepapred. The slides were de-paraffinized and were subjected to staining.

One slide was stained with Harris haematoxylin and Eosin stain mounted in DPX(di-n-butyl pthalate in xylene) and observed under oil immersion objective of a binocular compound light microscope(Olympus).

Other slide was stained with freshly prepared Acridine orange dye (figure 6).Acridine orange staining procedure:- Staining solution(100ml) was prepared from 1 mL of 1% aqueous stock solution by diluting 99 ml of distilled water. Right proportion of 1 % acetic acid was added to the acridine orange solution to attain the pH at 4.5. The staining method was followed as described by Bertalanffy et al[8].

Step 1 - Hydration in regressive changes of alcohol

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Step 2 - Distilled water -- 2 minutes

↓

Step 3 - 1% acetic acid for 5 dip

↓

Step 4 - Distilled water for 2 minutes

↓

Step 5 - 0.01% Acridine Orange staining solution for 5 minutes

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Step 6 - Phosphate buffer solution (PBS) at pH 6 for 1 minute

↓

Step 7 - 0.01M Calcium chloride solution for 2 minutes

↓

Step 8 - Phosphate buffer solution (PBS) for 1 minute

↓

Step 9 - Slides mounted in PBS

0.01Mcalcium chloride solution was used to bring about the differentiation between ribonucleic acid (RNA) and deoxyribonucleic acid(DNA).[8]

A modification was done by rehydrating the tissue sections in regressive changes of alcohol 100 %, 80 %, 70%, 50%, followed by incubation for 24 hours at 4degrees in PBS. This modification was

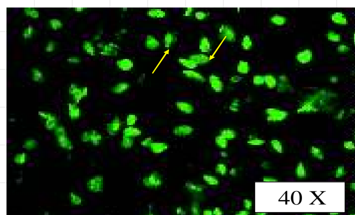
done in order to improve the penetrability of the fluorochrome into the formalin fixed paraffin embedded tissue sections. This automatically enhanced the binding of the dye with the DNA of the cell and improved the DNA analysis. Later the staining procedure was continued from the step 2.[9]

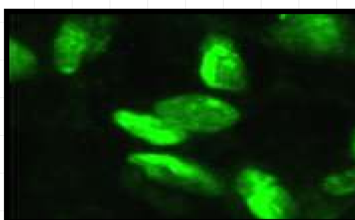
Slides were mounted with phosphate buffer solution(PBS).The stained sections were observed under 40X objective of BX41 Olympus fluorescent microscope using excitation filter 460-490nm and emission filter 520nm at position 2 and with an exposure time of 900ms. 100 cells were observed on each slide and the total number of Barr body positive cells were counted and the percentage of the same was calculated.

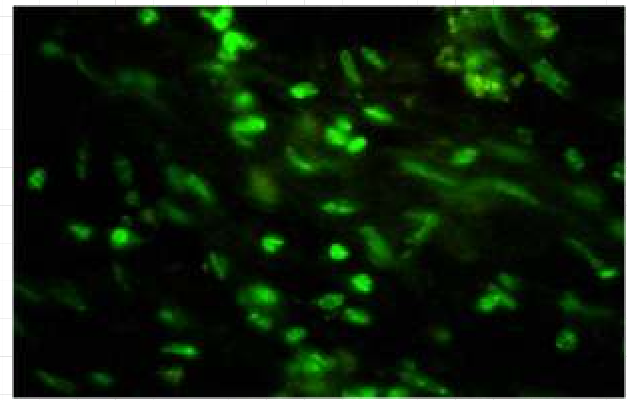
Results

In the present study, Acridine orange fluorescent microscopy showed the following results from the dental pulp tissue of male and female cases.

1. Among the 25 female samples given by the evaluator, 24 were diagnosed correctly as females (true positives) and the rest 1 female cases was diagnosed as male(false negatives).
2. Among the 15 male samples, 13 samples were correctly diagnosed as males(true negatives) and the rest 2 samples were diagnosed as females (false positives) (tablel)
3. In the female samples, the percentage of Barr body positive cells ranged from 19-48%.
4. In male samples, the percentage of Barr body positive cells ranged from 0-2%.
5. The majority of the female samples (17 samples) had a lower percentage of Barr body positive cells (19 to 35 %) and in only a few samples (8 samples) it was greater than 35%. None of the women showed less than 19 % Barr-body positive cells.
6. Female sample– Fibroblasts with oval nuclei, there was hyperchromatism, with the condensation of the Barr chromatin at the nuclear periphery with a variable number of cells, with collagen fibres interspersed in between the fibroblasts (figure 7 & 8) .
7. Male sample—Fibroblasts with hyperchromatic nuclei, with no further condensation of chromatin in the nuclei, with collagen fibres present in between the fibroblasts (figure 9)
8. With the younger age group we were able to find more number of fibroblasts and less of the fibres and as compared to the elderly age group in which low cell density more number of collagen fibres were present.







		Observations by the evaluator		
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Observations by the principal investigator	FEMALE	24 (a) True-positives	2 (b) False positives	26
	MALE	1 (c) False negatives	13 (d) True negatives	14
	TOTAL	25	15	40

- **Mean:-** 28.46
- **Sensitivity:-** $\frac{a}{(a+c)}$ = 96%
- **Specificity:-** $\frac{d}{}$ = 87%

Discussion

The identification of the deceased has always been of paramount importance to the human society. Determination of sex helps the forensic investigator to resolve many cases of assault, thefts and sexual offences.

We often encounter cases of mass disasters, mass transportation accidents, murder or injury from assault, where a single tooth is the sole material available for evaluation. In these situations, even a single tooth can serve as a valuable source of information for the identification of the deceased individual.[6]

Various features of the teeth such as morphology of the teeth and advanced DNA extraction methods from the dental cells have been used to distinguish the sex of an individual. Both of these methods have their own limitations.[5]

The present study aimed at exploring an intermediate method , i.e. the histological method. The advantage of which is that it can be easily performed with minimal requirement of heavy equipments such as used in PCR (Murakami et al.) and LAMP method (Nogami et al.).[3] The microscopic examination of the cells from the tooth pulp can reveal the presence of Barr bodies in females, thereby proving

to be a reliable source for the identification of the sex of the individual[5].

Barr body, also called as sex chromatin or X chromosome can be described as heteropyknotic, basophilic, condensed intranuclear structure seen in mammalian somatic cells during the interphase[7].

Sex-chromatin was discovered in 1949 by two Canadian researchers, Murray Barr and Edward Bertram. They had initially noticed a dark staining blob of material in the nucleus of the nerve cells of female cats which was absent in males[10].

It was subsequently determined that the little stained body was one of the X chromosomes, an inactive one that remained coiled and condensed, hence could be seen and later it was visible in human female somatic cells as well.[11]Various tissues such as buccal mucosal cells, hair, white blood cells are analysed for sex identification.[3]

Barr body is present in a large proportion of nuclei of female origin and absent in male nuclei. It measures about 0.8 to 1.1 micrometers in diameter and can be plano-convex, biconvex, triangular spherical or rectangular in shape. [7]

Nuclear dyes such as cresyl violet, thionin and haematoxylin are known to stain the Barr bodies intensely. (Lindsay and Barr, I955), stated that that they are composed of material similar to that of the chromosomes and contain DNA.[12]

Mechanism behind the formation of Barr bodies:-One of the X chromososmes in female is rendered inactive by the process of lyonization, is named after the scientist Lyon. In 1961, Lyon outlined the X inactivation or what is commonly called as the Lyon hypothesis which states that: Only one of the X chromosomes is genetically active; the other X of either maternal or paternal origin undergoes heteropyknosis and is rendered inactive; inactivation of either the maternal or the paternal X occurs at random among all the cells of the blastocyst on or about the 16th day of embryonic life; and inactivation of the same X chromosomes persist in all the cells derived from each precursor cell.[7]

This inactivation is initiated from the X inactivation centre located near the centromere and the gene XIST (X inactive specific transcript) is located in the X-inactivation centre, expressed only by the inactive X chromosome.[10]

Barr bodies can also be seen in unstained cells and in living cells by means of phase-contrast microscopy. This was described by James in 1960. Also they were visible in fibroblast cultures obtained from human skin biopsies.[12]

According to the study done by Klinger in 1957 in amnion and connective tissue, indicated three possible positions of Barr body in the nucleus,:-

a) 61.8 % - At the nuclear periphery,

b) 23.2% - Lying against a nucleolus,

c) 9.2% - Free in the cytoplasm

Most of the investigators only take those cells into account in which the Barr body is located at the nuclear periphery which underestimates the true incidence of the actual count of Barr body positive cells. [12]

In the present study, fibroblasts from the pulp tissue were evaluated for the presence of the Barr bodies. Barr bodies within the pulp tissue can be observed with most of the nuclear stains such as haematoxylin and eosin, papanicolaou, fuelgen, cresyl violet, aceto-orcein, carbol fushin and fluorescence.[3] Fibroblast cells in which the Barr body was visible at the nuclear periphery were taken into account.

The application of fluorescent techniques to determine the nucleic acid concentrations in examining desquamated epithelial cells is a common procedure. Sectioned material, however, is seldom used for such procedures.

In the present study, Acridine orange being a chromatin specific fluorescent dye, was used to stain the histological sections of the dental pulp. When modification was done in the staining procedure, it showed crisp staining of the nucleus and a good contrast was observed between the chromatin and Barr body.

The modification was done in order to reverse the cross linking in the DNA and proteins produced by formaldehyde. This improved the penetration of the dye into the nucleus and thereby improved the DNA analysis. [13]

The results of the present study demonstrate that acridine orange dye reliably stains the aldehyde fixed sections and has a simple staining procedure.

Various conditions such as weather, temperature, humidity and the duration for which the tooth is held in the environment unpreserved which will influence the process of decomposition of the dental pulp tissue.

A number of studies have been done by various authors who have placed the extracted tooth under various natural and artificial conditions for variable periods of time. They found out that the pulp tissue was affected under these conditions and this further affected the determination of sex by the pulp tissue.(table-II)

The results obtained in our study are similar to those reported by Yunis and Chandler (1979), in which approximately 30% of female fibroblast nuclei were Barr body positive, with a range of 15-40%.[3]

Our results are also consistent with results of Das et al. who showed an average of 24.92% of female pulp cells to be positive for the presence of Barr body after 4 weeks time.[20]

Our results also match with that of Ivan Suazo et al. (2010) who found an average of 30 % female cells to be positive for the presence of Barr bodies after boiling the teeth at 200° .[3]

In the present study only healthy teeth were studied, however Barr in 1957 and Larson &Knapp in 1971 were able to observe the Barr bodies in polymorphonuclear neutrophils within the inflamed dental pulp tissue.[3]

The pulp tissue from male samples in our study showed 1-2% of cells positive for Barr bodies, whereas study done by Duffy et al. showed Barr bodies in a range of 0to 6% and almost similar results were given by Das et al in 2004.[20]

A possible reason for the finding of Barr body in males can be explained by the more or less darkly staining bodies called chromocentres present during the interphase. Such non-specific chromocentres are generally smaller than Barr bodies and have a less well-defined outline. The presence of such non-specific chromocentres, which are almost similar to Barr bodies in the male nucleus can be mistaken for the latter. [12]

Extracted tooth placed in various conditions	Seno, M and Ishizu, H.1973[14]	Whittaker et .al. 1975[15]	Seno,M. 1977[16]	Dange et. al 1978[17]	Ionesiy, A.G. 1980[18]	Duffy,J. 1991[19]	Das et. at 2004[20]	Sauzo.et.al. 2010[3]
Room temperature				Analysed gender even after 4 yrs	Decrease in visibility of Barr body in 1year.			
Bacterial decomposition	Barr body visible 5months after death.	5weeks postmortem					4weeks post-mortem.	
Temperature						Possibility for determining gender after heating tooth at 100 °for 1 hour		Visibility of Barr body after heating the tooth at 200°, 400°.
Buried in mud and sand			Barr body visible after burial of tooth for 1 month					
Running water						Destruction of cellular structures of pulp tissue in 3days		
Refrigeration (-4° to -8°)					Possibility of finding Barr body until 30 days			

Table II-Various studies taken up by various researchers who analyzed the presence of Barr bodies after keeping the extracted teeth under various conditions.

The present study was undertaken in search of such a technique which combined rapidity, reliability and specificity in the determination of sex.

The present conducted study was a single blind study in which the principal investigator was unaware of the details of the tooth samples provided by the evaluator. The results of our study were quite appreciable with a sensitivity of 96% and a specificity of 87%.

To conclude, the method of single blind study has reinforced the validation of identifying the presence of Barr bodies in otherwise unknown pulp tissue.

Conclusion

It poses a challenge for the forensic investigator to determine the sex of the victim when the body is destructed or mutilated beyond the scope of identification by any other method.

In such cases, teeth can serve as a valuable source of information for the forensic odontologist in determining the gender of the deceased.

The observations of our study and the statistical analysis make the pulp tissue a significant source in determining the sex of the individual. Acridine Orange dye used in fluorescent microscopy proved to be a reliable stain to identify the sex of the individual from the dental pulp tissue.

Further research and evaluation to increase the specificity of the study needs to be done with a larger sample size to substantiate the findings.

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