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Comparative Study On The Effect Of Some Histological Techniques On The Quantitative Morphometric Analysis

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Abstract

Quantitative morphometric studies are commonly used nowadays in histological and pathological labs and researches. The aim of the study was to evaluate the possible effect of the histological techniques on the morphometric results and determination of correction coefficients of morphometric parameters in dependence on the histological procedure used.

The organs and cells subjected to study were ; human RBCs (lack of nuclei), albino rats liver cells (active cells) and albino rat uterus (for gross study and its muscles and fibers content). Three techniques were selected; fresh cells (blood smear and liver cell print), frozen technique and paraffin sections fixed in neutral buffered formol (common histological technique). Quantitative morphometric analysis parameters selected were diameter, perimeter, area and area percent. The obtained results were statistically analyzed by using student paired t-Test. The study showed statistically significant changes in quantitative morphometric results under the effect of histological techniques used. Frozen technique increased the morphometric results , while paraffin technique decreased them when compared with those of fresh data. Also the morphometric data of gross area and perimeter of organs affected by their fibrous elements. The study concluded that all quantitative morphometric results must be corrected by a coefficient factor which depends on the organ and technique used before analysis and evaluation of data.

Introduction

In the last few years, as a result of introduction of computer vision (Image Analyzer), the quantitative histological techniques and interactive morphometry study becomes common on many histological and pathological research works.

The reproducibility of the measure - ments depends on the tissue or cells and staining methods. A number of tech - nical factors may cause random errors, such as quality of the slides, magnify - ication, definition of the cells to be measured and measuring protocol.

Having quantified cell and tissue features, multivariate analysis may result in a better discrimination of two or more groups under study and can provide important diagnostic and prognostic information.

Quantitation requires skill in object selection and the quality control of the whole measuring system. The use of quantitation as a black box can result in dramatic errors.

The quantitation subdivided into 3 subdivisions, morphometric analysis (e.g., count, distance, diameter, perime - ter, area, area percent volume etc.,), color densitometric analysis (e.g., evaluation of histological stains, immuno-histological reactions or enzymatic activities colors as in depth or distribution) and the kinetic analysis (e.g., velocity, types of motion, amplitude, pattern of motion etc.).

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Tissue spread, frozen and paraffin techniques are commonly used for preparation of histological or patholo - gical slides. In fresh unfixed histology - cal sections (e.g., blood film, connective tissue spread, vaginal smear etc.,) direct analysis of cells may give more reliable information than from frozen or paraffin sections. Morphometric analysis of tissue or cells can be affected by various factors such as, change in temperature (as in freezing or paraffin sections), tissue water content (increased in frozen and low in paraffin techniques), or exposure to chemicals. The present study aimed to study the effect of histological techniques on the quantitative morphometric results.

Materials and Methods

The present study was done on human RBCs, adult albino rats hepatocyte, and adult albino rats uterus. Neutral buffered formol was used as a fixative. All paraffin and frozen sections were cut at 10 micrometers thickness. Hematoxyline and Eosin staining technique and Mallory trichrome stain were used in the study.(Drury and Wallington 1980).

Study of human red blood corpuscles diameters and areas:

- Ten fresh blood film were prepared by spread method without fixation, staining or mounting.
- Ten frozen and 10 paraffin slides were prepared from normal human full term placenta.
- Frozen and paraffin sections were stained, dehydrated, cleared and mounted in DPX.

Study of albino rats hepatocytes for cellular and nuclear diameters and areas:

- Ten fresh sections of hepatocytes were obtained by cell print method and stained without fixation, dehydration or mounting.
- Liver biopsy was taken for preparation of 10paraffin and another 10 section.
- Frozen and paraffin slides were stained, dehydrated, cleared and mounted.

Study of albino rat uterus for gross perimeter and area, uterine cavity (perimeter and area), and area percent of uterine collagen and muscle fibers.:

- Ten frozen and another 10 paraffin sections of uterus were prepared, stained, dehydrated, cleared and mounted in DPX.

The quantitative morphometric analysis studies were done by using SupeEye Image Analysis System – HeidiSoft Co. - Egypt. The obtained results were statistically analyzed by using pair student T-test.

Results

- Human red blood corpuscles morphometric results: are illustrated in Tables 1 -2 and Figures 1 to 3 and Figure 9.

(Table 1):The effect of histological techniques on the diameter of human RBCs

	Fresh	Frozen	Paraffin
Mean(µm)	6.84	7.50	5.47
SD	0.67	0.51	0.73
SEM	0.067	0.11	0.16
Min	5.14	6.87	4.11
Max	8.22	8.52	6.90
T-Test		Fresh vs. Frozen	Fresh vs. Paraffin
		5.59E-5	5.69E-13
p-value		p<0.001	p<0.001
Significance		Sign. Inc.	Sign. Dec.
Change (%)		8.85	20.04

* SD= Standard deviation SEM= Standard error of mean
Min= Minimum value Max= Maximum value
T-Test=Student t-Test (pair) Significant = $p<0.05$
Sign. Inc.=Significant increase Sign. Dec.=Significant decrease
NS=Non significant change
Change(%)= abs(100 – (Mean ValueXX / Mean Fresh value)*100)

Table –2 The effect of histological techniques on the area of human RBCs

	Fresh	Frozen	Paraffin
Mean(μm^2)	36.29	46.40	22.75
SD	5.59	4.78	3.72
SEM	1.25	1.07	0.83
Min	26.13	38.33	16.60
Max	44.27	53.460	31.03
T-Test		Fresh vs. Frozen	Fresh vs. Paraffin
		3.6E-07	5.5E-11
p-Value		p< 0.001	p< 0.001
Significance		Sign. Increase	Sign. Decrease
Change (%)		27.86	37.30

Figure – 1- Computerized photomicrograph of of normal human blood film

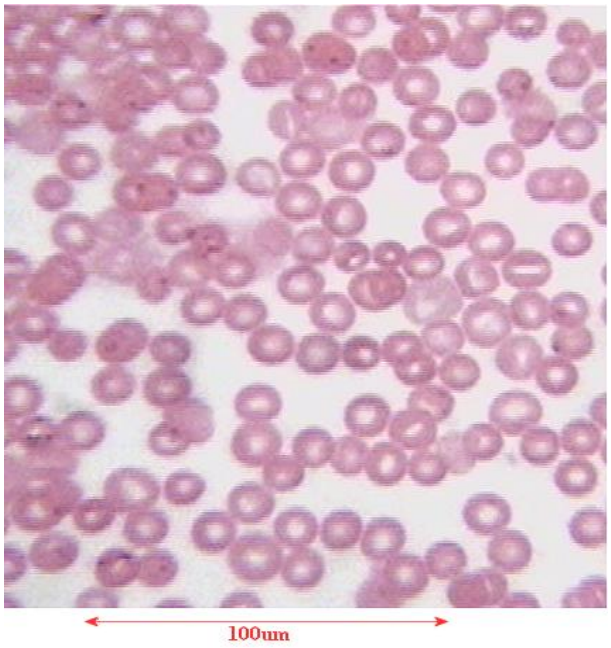


Figure – 2-Computerized photomicrograph of human normal full term placenta prepared by frozen technique (Mallory trichrome stain)

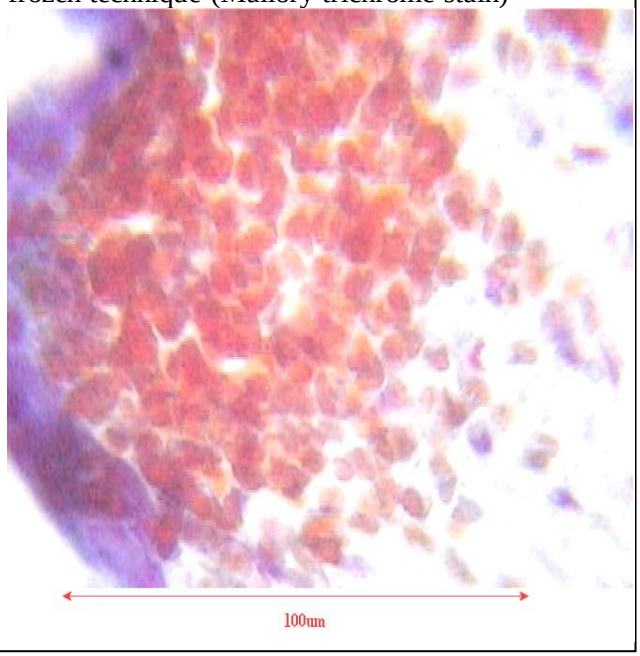


Figure3-Computerized photomicrograph of normal human full term placenta prepared by paraffin technique (Hx and E. stain)

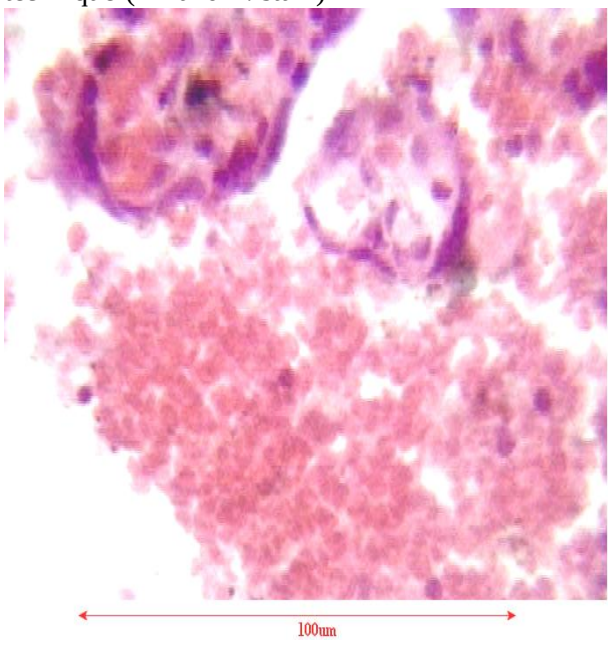


Figure 4-Computerized photomicrograph of adult albino rat hepatocytes prepared by cell print technique (Mallory trichrome stain)

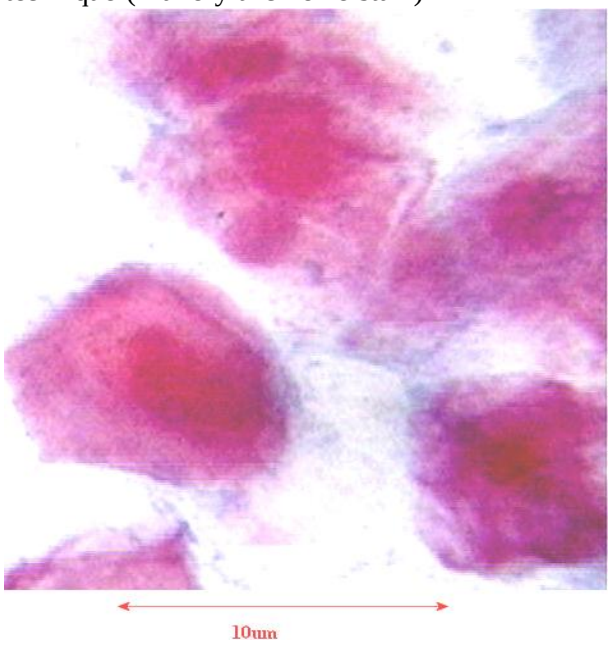


Figure 5-Computerized photomicrograph of adult albino rat hepatocytes prepared by frozen technique (Mallory trichrome stain)

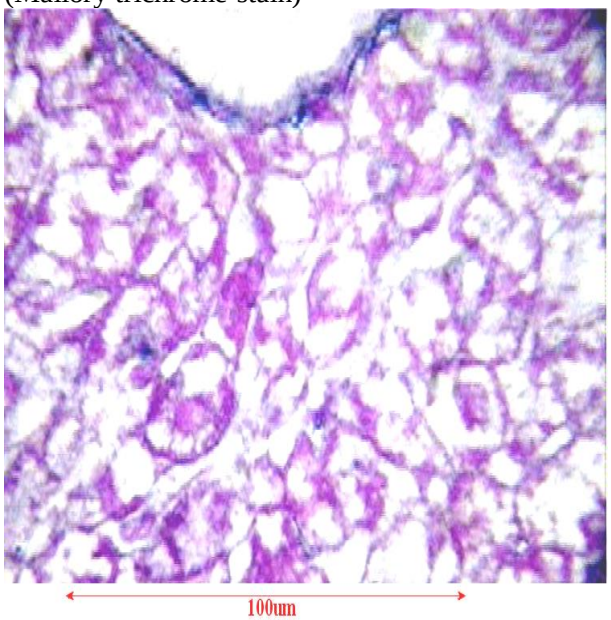


Figure 6-Computerized photomicrograph of adult albino rat hepatocytes prepared by paraffin technique (Hx and E. stain)

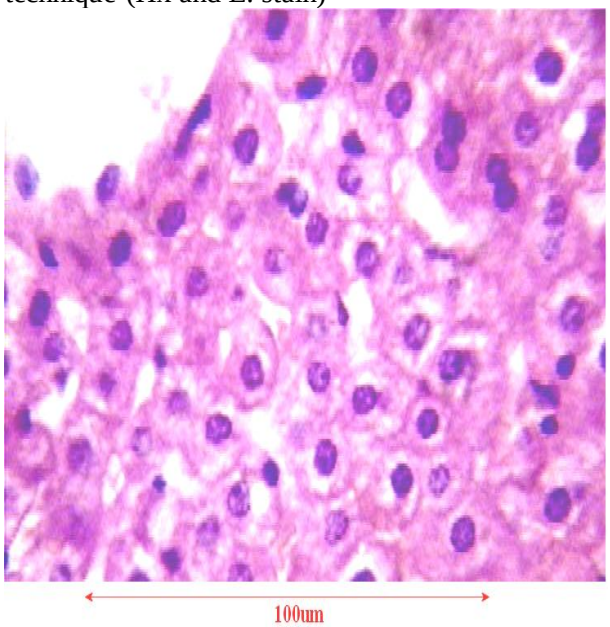


Figure 7-Computerized photomicrograph of transverse section in normal female albino rat uterus prepared by paraffin technique (Mallory trichrome stain)

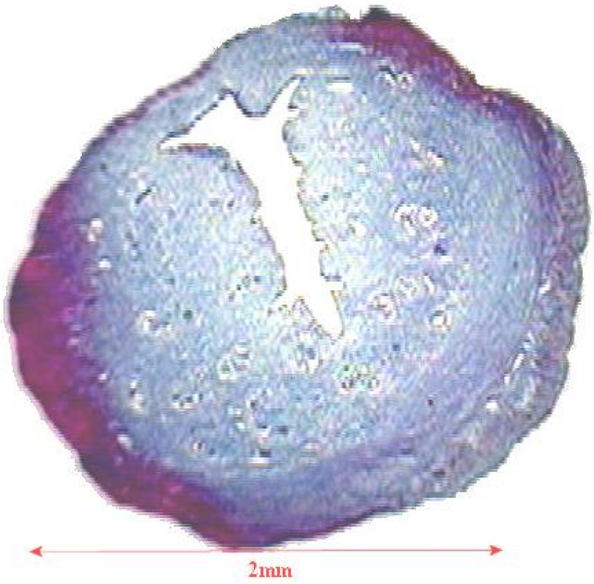


Figure 8-Computerized photomicrograph of transverse section in normal female albino rat uterus prepared by frozen technique (Mallory trichrome stain)

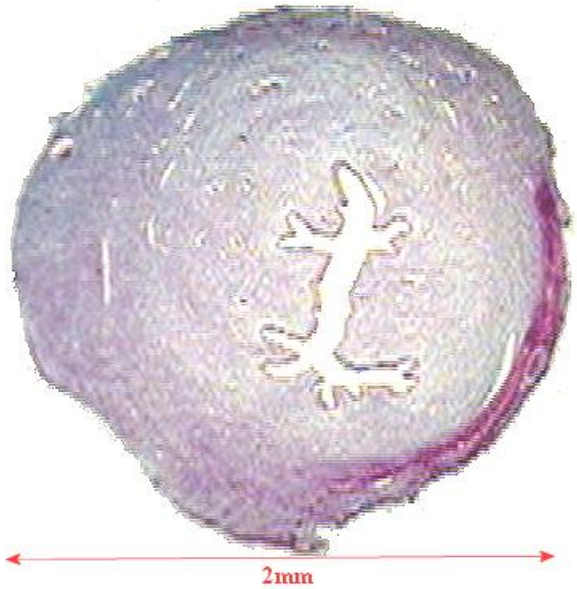
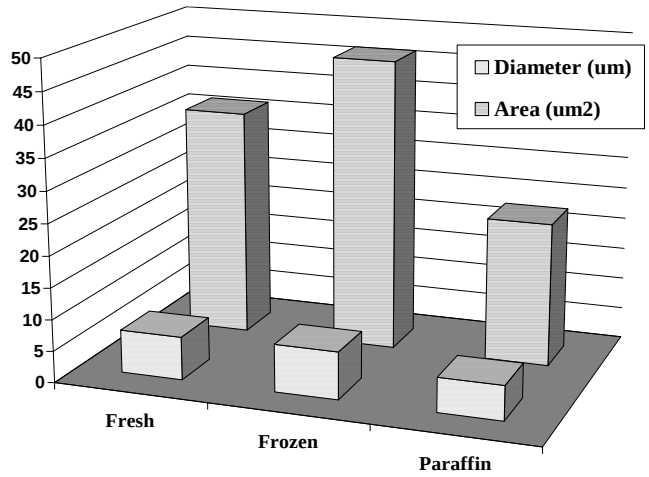


Figure –9 The effect of histological techniques on diameter and area of human RBCs



- **Albino rats hepatocytes morphometric results:**

The effect of histological techniques on the morphometric results of albino rat hepatocytes are summarized in tables 3 - 4 and figures 4-6 and figures 10-11

Table-3The effect of histological techniques on rat hepatocytes cell and nuclear diameters

	Fresh		Frozen		Paraffin	
	Cell	Nucleus	Cell	Nucleus	Cell	Nucleus
Mean	10.61	5.09	13.09	5.81	7.31	3.47
SD	2.64	0.7	3.26	0.79	0.98	0.87
SEM	1.98	1.13	2.4	1.29	1.80	0.83
Min	6.8	3.67	8.38	4.19	5.26	1.81
Max	15.92	6.11	19.64	6.99	9.37	5.1
t - Test			Fresh vs. Frozen	Fresh vs. Frozen	Fresh vs. Paraffin	Fresh vs. Paraffin
			0.012	0.004	6.15E-06	1.27E-07
p- value			p<0.05	p<0.005	p<0.001	p<0.001
Sign.			Sign. Inc.	Sign. Inc.	Sign. Dec.	Sign. Dec.
Change%			23.37	14.29	31.12	31.78

Figure-10-The effect of histological techniques on rat hepatocytes cell and nuclear diameters

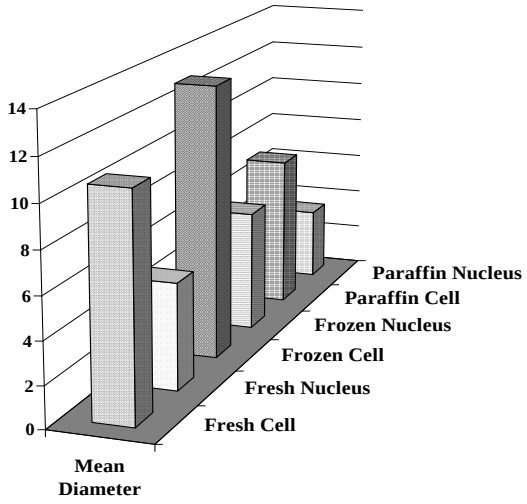
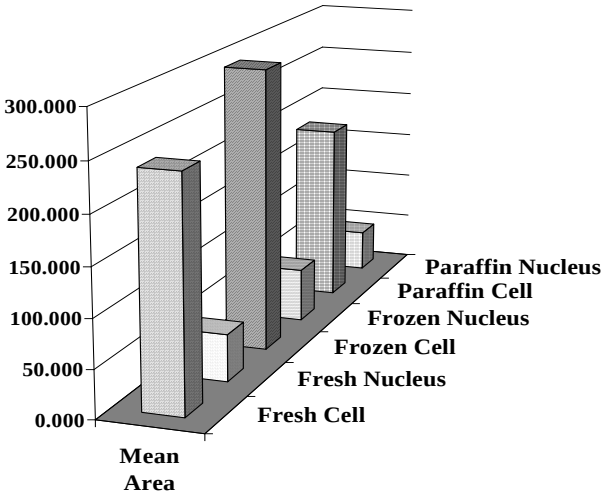


Table-4- The effect of histological techniques on rat hepatocytes cell and nuclear areas

	Fresh		Frozen		Paraffin	
	Cell	Nucleus	Cell	Nucleus	Cell	Nucleus
Mean	239.3	49.31	295.21	56.35	187.68	43.61
SD	68.77	13.04	84.83	14.90	53.93	11.53
SEM	76.67	9.64	94.57	11.01	60.12	8.52
Min	112.46	26.5	138.73	30.28	88.2	23.43
Max	345.94	74.43	426.77	85.07	271.32	65.83
T-Test			Fresh vs.	Fresh vs.	Fresh vs. Paraffin	Fresh vs. Paraffin
			0.028	0.120	1.2E-02	1.51E-01
p- value			p<0.05	p>0.05	p<0.05	p>0.05
Sign.			Sign. Inc.	NS	Sign. Dec	NS
Change			23.37	14.29	21.57	11.56

Figure –11-The effect of histological techniques on rat hepatocytes cell and nuclear areas



- **Albino rats uterine morphometric results:**

The effect of histological techniques on the gross perimeter and area, uterine cavity (area and diameter), and area percent of uterine collagen and muscle fibers are summarized in tables 5 - 7 and figures 7-8 and figures 13-16

Table-5-The effect of histological techniques on the perimeters of uterus and uterine cavity of the albino rats

	Lumen		Uterus	
	Paraffin	Frozen	Paraffin	Frozen
Mean(um)	2312.57	1798.59	6039.32	5360.95
SD	250.76	125.50	573.44	370.70
SEM	79.30	39.69	181.34	117.23
Min	2001.39	1569.72	5381.52	4910.53
Max	2776.32	1993.88	7023.32	5903.92
T-Test		1.72E-05 Frozen vs. Paraffin		5.64E-03 Frozen vs. Paraffin
p-Value		p<0.001		p<0.01
Sign		Sig. Dec.		Sig. Dec.
Change%		22.23		11.23

Figure-12-The effect of histological techniques on the perimeters of uterus and uterine cavity of the albino rats

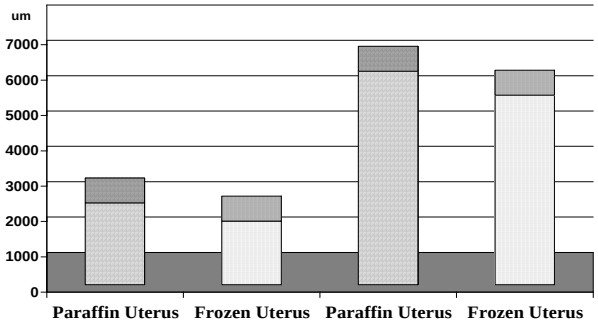


Table 6-The effect of histological techniques on the areas of uterus and uterine cavity of the albino rats

	Lumen		Uterus	
	Paraffin	Frozen	Paraffin	Frozen
Mean(um^2)	466948.34	416174.07	1484663.08	1385010.00
SD	20692.96	26651.98	30261.08	7276.83
SEM	6543.69	8428.10	9569.40	2301.14
Min	431086.00	384858.00	1406464.00	1377098.00
Max	494772.00	464875.00	1510042.00	1399983.00
T-Test		1.57E-04		7.37E-09
		Frozen vs. Paraffin		Frozen vs. Paraffin
p-Value		p<0.001		p<0.001
Sign		Sig. Dec.		Sig. Dec.
Change%		10.87		6.71

Figure-14 -The effect of histological techniques on the areas of uterus and uterine cavity of the albino rats

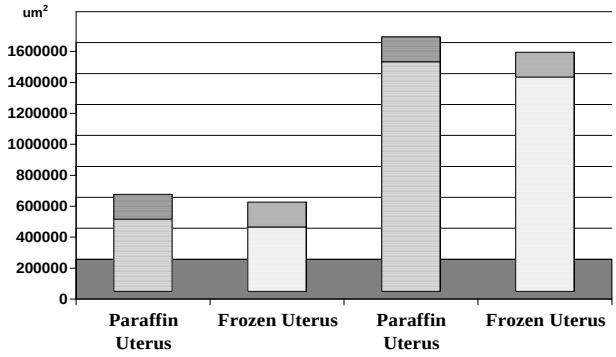


Table-7The effect of histological techniques on the uterine collagen and muscle fibers percentage areas of the albino rats

	Paraffin			Frozen		
	Muscles	Collagen	M/C Ratio	Muscles	Collagen	M/C Ratio
Mean%	0.30	0.31	0.97	0.23	0.47	0.50
SD	0.01	0.03	0.13	0.01	0.05	0.07
SEM	0.00	0.01	0.04	0.00	0.02	0.02
Min	0.28	0.26	0.82	0.21	0.38	0.39
Max	0.33	0.35	1.16	0.25	0.57	0.60
T-Test				Frozen vs. Paraffin	Frozen vs. Paraffin	Frozen vs. Paraffin
				8.2E-10	2.8E-07	4.7E-09
p-Value				p<0.001	p<0.001	p<0.001
Sign				Sig. Dec	Sig. Inc.	Sig. Dec
Change %				23.99	49.44	48.95

*Mean%= (collagen or muscle fibers area) / whole uterine area) *100
*Comparison was done on frozen muscle vs. paraffin muscle .
* M/C ratio= XXX muscle / XXX collagen
*M/C Comparison was done on frozen M/C vs. paraffin M/C

Figure-14 The effect of histological techniques on the uterine collagen and muscle fibers percentage areas of the albino rats

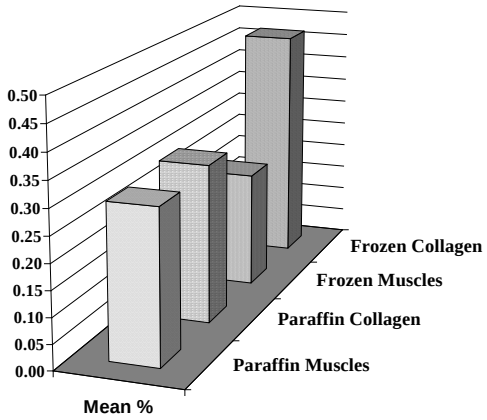
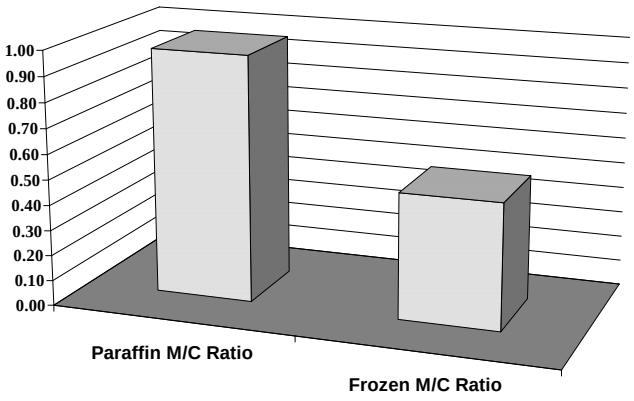


Figure-15 The effect of histological techniques on the ratio uterine muscle to collagen fibers ratio of the albino rats



Discussion

The present study was planned to demonstrate the possible effect of the histological technique used on histol - ogical Quantitation. Measuring of hum - an red blood corpuscles diameters and areas in fresh preparation was consid - ered as a starting point in the study, and to evaluate the image analyzer measuring quality. The obtained data were in an acceptable normal control range (RBCs diameter was 6.84±0.66 um, and area 36.29±5.59 um^2).

The diameter and area significantly increased after freezing technique ($p<0.001$) (7.50um ±0.51 and 46.4um^2 ± 4.78 respectively), and decreased in paraffin technique (5.47um ±0.73 and 22.75 um^2 ± 3.71 respectively). The variation in RBCs diameters was 8.85% in frozen and -20.04% in paraffin, while area was affected by 27.86% in frozen results and -37.30% in paraffin.

Study of rat hepatocytes cell diameters showed statistically signify - cant changes (7.31um ± 2.64, 13.09um ±3.26 and 10.61um ±0.98, in fresh, frozen and paraffin respectively).

Similar finding was observed in hepatocytes nuclear diameter (5.09um ± 0.7, 5.81um ±0.79 and 3.47um ±0.87, in fresh, frozen and paraffin respectively). Also, the mean hepatocytes cell area

was changed (239.3um^2 ±68.77, 295.21 um^2 ± 84.83, and 187.68 um^2 ± 53.93 in fresh, frozen and paraffin respectively). While the mean hepatocytes nuclear area was not statistically affected , $p>0.05$, (49.31um^2 ±13.04, 56.35 um^2 ± 14.90, and 43.61 um^2 ± 11.53, in fresh, frozen and paraffin respectively)

The frozen and paraffin histological techniques also caused a change in, the gross uterine perimeter and area, and uterine wall (perimeter and area). The mean paraffin uterine area and perim -eter was 1484663.08 um^2 ± 30261.08 and 6039.32 um ± 573.44, respectively) while in frozen was 1385010.00 um^2 ± 7276.83 and 5360.95 um ± 370.70, respectively). Regards uterine cavity, the mean paraffin area and perimeter was 466948.34 um^2 ± 20692.96 and 2312.57 um ± 250.76 , respectively) while in frozen was 416174.07 um^2 ± 26651.98 and 1798.59 um ± 125.50 , respectively).

During the evaluation of total uterine (collagen, muscle fibers and their ratio) as area percent, a statistically significant variation was observed betw -een paraffin and frozen uterine sections. In paraffin section percentage of muscle fibers was 30% ± 1% , collagen fibers 31% ± 3% and the ration of muscle fibers to collagen fibers was 0.97 : 1.0, while in frozen sections the muscle fibers percent decreased to 23% ± 1% , the collagen fibers percent increased to 47% ± 1% and the ratio of muscle to fibers was 1 : 2

The change in cell morphometric analysis is agreed with researches of Pentilla *et al*., (1975), Schmid-Schonbein *et al*., (1980), Gerdes *et al*., (1982), Hanstede and Gerrits (1983), Reith *et al*., (1984) , Wegiel et al., (1989), Miller and Meyer (1990) and Gilbert and Parmley (1998).

Pentilla *et al*., (1975), stated that, during fixation, tissues commonly change in volume and the mechanisms involved are ill-understood, and various factors have been suggested, including inhib -ition of respiration, changes in memb -rane permeability or in ion transport through the membranes. The subseq -uent dehydration and embedding will also bring about further changes in volume. Ideally, these changes should cancel each other out to give no net change. Tissues fixed in formaldehyde and embedded in paraffin wax shrink by 33 per cent. The nuclei in frozen sections are usually bigger than those of the same tissue which has been subjected to conventional preparation, but these changes are relatively small compared to other organelles. Prolonged fixation in formalin can give rise to secondary shrinkage. Injured cells swell or shrink to a different extent to normal cells in chemical fixatives.

Pentilla et al., (1975), also reported that some intracellular substances such as collagen swell when they are fixed and besides these volume changes which occur during fixation, the net change in volume of the various compo -nents of tissue should be considered through to the section on the slide. This is of importance when slides are compared with living tissue, for example in histometric studies.

Schmid-Schonbein *et al*., (1980), studied the possible artifacts due to preparation of the cells for transmission electron microscopy, with a detailed comparison with light microscopy. The quantitative morphometric parameters of leukocyte functions were include the diameter, volume, and membrane area of the cells and their nuclei in the undeformed state. A stereological meth -od was used to obtain these quantities from transmission electron microscopy of random sections through human white blood cells (neutrophils, lympho -cytes, monocytes, and eosinophils). The

results showed that undeformed white cells in isotonic solution are spherical with many membrane folding and had a significantly smaller diameter than that measured on blood smears and a method of chemical fixation was empl - oyed so that the shrinkage due to fixation of the cells was below the resolution of light microscopic measure -ments. Further, it was shown that all leukocytes, including lymphocytes, had much more membrane area than was needed to cover their volumes, and this membrane area remained constant when the cell was hypotonically swollen .

Gerdes *et al*., (1982) studied the morphometric changes occurred in an isolated cardiac myocytes through the entire procedure after fixation with iso-osmolar glutaraldehyde and investigated these changes, adhering of the cells to glass cover slips of Sykes Moore cham - bers and photographed after each step of processing for transmission electron microscopy. The cellular dimension changes were determined by tracing individual isolated myocytes after each step of the procedure with a sonic digit - izer. Significant cell volume changes occurred after osmium (16% swelling), post osmium wash (10% swelling), and uranyl acetate (25% shrinkage).

Hypertonic aldehyde solutions resulted in cellular shrinkage during fixation not found with isotonic solutions. Changes in cell cross-sectional area rather than length were largely responsible for altered cell volumes during any given phase of processing. The results indicated that, although cell volume changes occur during processing, final cell dimensions of embedded cells were not different from unfixed cells.

Hanstede and Gerrits (1983), described the morphometric changes of the liver samples, in the course of fixation, dehydration, infiltration and embedding in different mixtures of water-soluble plastics (glycolmeth - acrylate (GMA) and the commercially available material JB4). Buffered formaldehyde fixation did not produce significant morphometric changes in the liver specimens. Dehydration obviously affects the volume of the liver specimen (linear shrinkage about 9.3%). The dehydration is followed by an infiltra - tion phase. During this phase a slight swelling (linear, 2-5%) occurs. Correction factors must be used in morphometric and stereological investigations.

Reith et al., (1984) studied the influence of perfusion versus immersion fixation with cacodylate buffered gluta - raldehyde, osmium or glutaraldehyde immersion fixation with two vehicles, phosphate and cacodylate on cellular and sub cellular structure of animal hepatocytes. There were 15% increase in the volume of hepatocytes (mainly their cytoplasm), and 30% increase after immersion fixation in osmium in comparison to perfusion fixation. The mitochondrial enlargement was particu - larly displayed in the organelles, average profile area, being more than doubled (211%) in immersion fixation. Similarly high profile enlargements (150%) were also found after osmium immersion fixation. Changes in nuclei were minor compared to the cytoplasm.
Wegiel et al., (1989) reported that the estimation of the volume of the rat substantia nigra and striatum during the first half year of life fixed in 8% formaldehyde in at 20 C^0 for 48 hrs produced rapid increase of the brain weight and volume up to 52% of that of the fresh brain followed by slow decrease of brain weight of about 1-3%/24 hrs. Dehydration in ethyl alcohol produced violent decrease of brain volume and weight (from 32% up to 39% of the fresh brain weight). Clearing

in methyl bensoesane increases again the brain weight by a few percentage. So the histological procedure causes error size, more pronounced in fetal rat brain and in brain of 1-2-day-old rats.

Miller PL and Meyer TW. (1990); studied the effect of tissue preparation on glomerular volume in normal rats and the values for glomerular volume obtained in paraffin-embedded tissue were approximately 40% lower than values for glomerular volume obtained in methacrylate-embedded tissue from the same kidneys. The morphometric studies showed reduction in glomerular volume in immersion-fixed tissue associ -ated with lowered values of peripheral capillary wall surface area and reduced mean capillary radius compared with perfusion-fixed tissue.

Gilbert and Parmley (1998); reported that, neutrophil cells which cryofixed or fixed in dimethyl sulfoxide-cryofixation -freeze-substitution processing were significantly rounder, 27-30% larger in cell volume than neutrophil cells which had fixed in glutaraldehyde and post fixation osmium tetroxide. The increase in cell volume in cryofixed cells did not appear to be due to abnormal cell swe - lling, since membranes, nuclear envel - ope, and mitochondrial cristae were more intact than in glutaraldehyde and post fixation osmium tetroxide cells. The morphometric data of the nuclear compartment was 22% smaller, while the cytoplasm (and its associated compartments) was 29% smaller in glutaraldehyde and post fixation osmium compared to cryofixed or fixed in dimethyl sulfoxide - cryofixation - freeze - substitution processing neutrophils .

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دراسة مقارنة عن تأثير استخدام بعض التقنيات النسيجية على التحليل الكمى الشكلى

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من الشائع استخدام الدراسات الكمية فى معظم معامل وأبحاث علمى الأنسجة والأمراض. والهدف من هذه الدراسة هو دراسة تأثير أستخدام بعض التقنيات النسيجية على نتائج التحليل الكمى الشكلى وأمكانية أيجاد معامل رقمى لتصحيح أخطاء النتائج. وفى هذه الدراسة, قد تم تحضير عينات من الدم البشرى , وكبد ورحم الجرذان البيضاء البالغة بواسطة تقنيات المسح النسيجى, والتبريد و التحضير بشمع البارافين وأستخدام بعض المواد الحافظة و الصبغات المناسبة . وقد تم قياس أقطار ومساحات ومحيط والمساحة النسبية لبعض الخلايا والأنسجة بواسطة جهاز تحليل الصور بالكمبيوتر. وبعد تحليل النتائج أحصائيا أظهرت الدراسة اللآتى:

أستخدام تقنية التبريد وأستخدام الصبغات يؤدى الى حدوث زيادة ذو دلالة أحصائية فى نتائج التحليل الكمى الشكلى بدرجات متفاوتة فى الأنسجة والخلايا.

أستخدام تقنية التحضير بشمع البارافين وأستخدام المواد الحافظة والصبغات يؤدى الى حدوث نقص ذو دلالة أخصائية فى نتائج التحليل الكمى الشكلى بدرجات متفاوتة فى الأنسجة والخلايا.

وجود تغييرات فى نتائج نسبة محتويات الأنسجة من العضلات والألياف عند تغيير التقنية النسيجية المستخدمة.

وتوصى الدراسة بأهمية تصحيح نتائج التحليل والدراسة الكمية طبقا للتقنية النسيجية المستخدمة والنسيج الذى تم دراسته قبل تقييم النتائج وتفسيرها.