IMPACTS OF CHRONIC MESTEROLONE INTAKE ON POSTMORTEM CHANGES IN HEART OF ADULT MALE ALBION RATS (BIOCHEMICAL AND HISTOPATHOLOGICAL STUDY)

BY

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ABSTRACT

Background: Anabolic androgenic steroids (AAS) use by professional and recreational athletes is increasing worldwide. **Aim of the work:** This study inspected the postmortem changes within the first 24 h in heart of Mesterolone-treated rats. **Material and Methods:** 120 adult male albino rats were divided into two groups; Group I (control): 80 rats, subdivided into two equal subgroups; Group II (Mesterolone) (40 rats): treated with 2.14 mg/kg B.W Mesterolone dissolved in 1ml corn oil. All treatments were orally gavaged once daily for twelve weeks. Rats were euthanized, and the heart specimens (ventricles) were collected at zero-time, 4, 12, and 24 h PM for biochemical and histopathological investigations. **Results:** Mesterolone significantly increased pH, MDA, ATP, ADP, and glycogen values as well as reduced the GSH and lactic acid levels when compared to the control groups. A postmortem time-dependent significant drop in cardiac muscles pH, antioxidant marker, ATP, ADP, and glycogen levels, contrariwise a significant rise in lipid peroxidation and lactic acid concentration were recorded in both control and Mesterolone groups. A progressive PM histological deterioration and collagen degradation were detected while; Mesterolone significantly increased the collagen fibers reaction in cardiac muscles. **Conclusion:** These findings point out Mesterolone exposure's modifying effect on the energy content, oxidative status, and histological architecture of cardiac muscles in the early PMI. Outcomes should be carefully considered when determining PMI in cases with a history of AAS abuse or patients pretreated with these medications for long durations or sudden death in young healthy adults.

Keywords: *Mesterolone; Androgenic Anabolic steroid; Postmortem changes; sudden cardiac death; Masson's trichrome stains.*

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INTRODUCTION

Accurate estimation of the postmortem interval (PMI) is a complex task in forensic casework, especially in homicide or unwitnessed death investigations. It assists in distinguishing antemortem pathology from postmortem artifacts. During the last sixty years, numerous methods have been advocated for the determination of PMI by multiple means. PMI was determined by using multiple methods as early and late postmortem changes, hangs in biochemical parameters after death, detection of microscopic changes that occurred in organs after death, and lastly the detection of DNA and RNA degradation rate and their relationship with PMI estimation ***(Acosta-Ampudia et al., 2022)***. Moreover, erroneous PMI can impact the course and outcome of criminal investigations ***(El-Noor et al., 2016).***

Anabolic androgenic steroids (AASs) are broadly used among young professional and recreational athletes for improving physical performance, gaining muscle mass and aesthetic purposes ***(Pomara et al., 2016)***.

Several case reports of sudden death in young athletes indicate a link between chronic AAS abuse and increased risk of arrhythmias and sudden cardiac death. Considering that cardiac adverse effects are related to the chronic consummation of AASs, and because of the high prevalence of AAS use among young adults, cases of sudden death among youthful adults have increased. Chemical–toxicological analysis is a fundamental tool to assess the link between sudden cardiac death and AAS misuse ***(Hernández-Guerraet al., 2019).***

However, the incidence of people dying from AAS abuse is underestimated and few studies have been reported**.** When confronted with a sudden death in a young athlete, the post-mortem examination should focus on the physical phenotype, such as muscular hypertrophy, gynecomastia, striae in pectoral or biceps muscle, testicular atrophy, and acne that may suggest AAS abuse and a detailed

examination of the heart ***(Esposito et al., 2021)***.

Mesterolone (17-beta-hydroxy-1 alpha-methyl-5 alpha-androstan-3-one) is one of the commonly used androgen receptor agonists as hormone replacement therapy in hypogonadism. It has a unique chemical structure differing from other AASs as it escapes ring aromatization into estrogen therefore it has no estrogenic side effects. Moreover, mesterolone has very low hepatotoxicity and confers oral activity that allows its oral administration ***(Asfour et al., 2021).*** The frivolous use of these anabolic steroids for improving one's bodily appearance or athletic performance may have serious health consequences of which the medical community must be aware. ***(Robles-Diaz et al., 2015).***

The data on the association between AAS use and postmortem changes in the heart is limited. Hence, the aim of the present study was to investigate the biochemical and histopathological changes in the cardiac muscle of rats within 24 h after death following chronic use of an anabolic steroid (Mesterolone).

MATERIAL AND METHODS

Drugs and chemicals:

- **Mesterolone:**

Purchased as 25 mg of tablet Proviron, each pack contains 2 strips of 10 white tablets. Each tablet contains 25 mg of mesterolone, manufactured by **(**Bayer weimar Gm bH& Co KG) 99427- Weimar, Germany

- **Corn oil:** It was obtained in the form of an oily solution as a vehicle agent for Mesterolone.

- Experimental design:

One hundred twenty adult male albino rats (10 weeks of age and 180–200 g of weight) were used. The animals were acclimated to the laboratory conditions for two weeks before starting the experiment. Rats were housed in metal cages and maintained at a controlled temperature of 21–24 ◦C with relative humidity (50–60%), a 12 h light-darkness cycle, and fed with a pelleted commercial ration and water ad libitum. All animals were treated in line with the National Institutes of Health (NIH) for the Care and Use of Laboratory Animals in scientific investigations. The experimental protocol was approved by the Ethics of Animal Use in Research Committee. (Zagazig University, ZU-IACUC) approval number: ZU-IACUC/3/F/207/2021

The rats were divided into 2 groups as follows:

<u>Group I</u> (control group):

This group consisted of 80 rats that were subdivided into:

Subgroup I A (40 rats):

Rats received a regular diet and tap water for 12 weeks to measure the basic parameters.

Subgroup I B (40 rats):

Each rat will be treated with 1ml of Corn oil (vehicle of Mesterolone) once daily by gavage for 12 weeks.

<u>Group II</u> (Mesterolone treated group) (40 rats):

Each rat will be treated with 2.14 mg/kg B.W of Mesterolone (high therapeutic dose) dissolved in 1ml corn oil once daily by oral gavage for 12 weeks. The high therapeutic dose was calculated according to the method of **Yusuf et al. (2020).**

Sampling:

After 12 weeks of treatment, all rats of all groups will be sacrificed at the same time by cervical dislocation and will be kept at room temperature (25 ◦C) for dissection to obtain the heart (ventricles). The specimens were obtained at four PM time points including 0 (immediately after death), 4, 12, and 24 h.

The collected hearts will be cut from the apex to the base into 2 halves. At each time point, the ventricles were divided into two groups: The first group was weighed and homogenized (10% w/v) in potassium phosphate buffer solution (pH 7.4) using a tissue homogenizer, and then centrifuged at 3000 rpm for 15 min. The resultant supernatant was used for pH measurement and biochemical analysis. The second group will be fixed in 10% neutral-buffered formalin for histopathological investigations.

I-Biochemical investigations

1- Measurement of pH:

The tissues' homogenate's pH was measured at room temperature using a pH-meter (Automatic biochemistry analyzer SAT 450) previously calibrated with two standards (pH 4.00 and pH 7.00). The degree of hydrogen

ion activity is ultimately expressed as pH level which generally ranges from 1 to 14. For each sample, the determinations were made in duplicate ***(Ono et al., 2019).***

2-Determination of oxidative stress markers:

- **Measurement of Malondialdehyde (MDA)**

Tissue MDA was assayed using Biodiagnostic kits (Egypt) according to the method described by ***De Leon and Borges (2020).*** Where MDA can react with Thiobarbituric Acid (TBA) and give pink colored trimethylene complex. MDA activity was expressed in nmol/ml.

- **Measurement of Reduced Glutathione (GSH) enzyme**

Double-sandwich enzyme-linked immunosorbent assay technique (ELISA) was used. The method is based on the reduction of 5,5dithiobis (2-nitrobenzoic acid) (DTNB) nm with glutathione to produce a yellow compound (Colormetric methods). The reduced chromogen is directly proportional to GSH concentration, and its absorbance can be measured at 405 nm ***(Chi et al., 2002).***

3-Measurement of Adenosine Triphosphate (ATP) and Adenosine Diphosphate (ADP)

Adenosine Triphosphate and adenosine diphosphate were measured in the heart homogenate by ELISA using rat ELISA kits obtained from Cloud-Clone Corp, (CCC, USA) (Cat. No. CEA349Ge and CEB069Ge 96, respectively) ***(Pani et al., 2014).***

4-Determination of glycogen:

The concentrations of glycogen were determined in the tissue homogenate by Colorimetric/Fluorometric method using Glycogen Assay Kit (ab65620) obtained from BioVision, Uk ***(Takahashi et al., 2020).***

5-Measurement of Lactic Acid

Lactic acid concentrations were measured in heart tissue byenzymatic colorimetric method (LOX / PAP) with lactate oxidase and 4-aminoantipyrine. The color intensity of the resulting red quinoneimine dye is directly proportional to the lactate concentration. It is measured at 546 nm ***(Sullivan et al., 2019).***

II-Histopathological examination

The collected hearts (ventricles) specimens were fixed in 10% neutral buffered formalin for 48 h, dehydrated in graded ethanol (70%–100%), cleared in xylene, impregnated and embedded in paraffin, sectioned at 5.0 µm thick tissue sections, stained with hematoxylin and eosin (H&E) and Masson's trichrome stains, and examined microscopically for recording any histopathological alterations ***(Suvarna et al., 2018).***

III-Morphometric study:

Quantitative morphometric measurements were achieved by using the Leica QWin 500 image analyzer (Leica Ltd., Cambridge, UK). The area percent of collagen fibers stained sections was measured. Heart slides were examined by light microscope at 40x, using ToupView software version 1.0 to measure the intensity of collagen fibers staining (blue color). Using the interactive measure menu, 5 non-overlapping fields were analyzed per section for each animal, later; the means were statistically analyzed ***(Chen et al., 2017).***

IV-Statistical analysis

The collected data were expressed as Mean+ SD. The statistical analysis was done by Epi-info statistical package program version 6.04d, January 2001. The following statistical methods were used: One-way analysis of variance (ANOVA or F-test), Paired by paired t-test: for comparison of means of one group at different time intervals, and Leastsignificant difference (LSD). For all these statistical tests, P value of >0.05 = Non-Significant, P value of <0.05 = Significant (*), P value < 0.01 and < 0.001= Highly Significant (**) **(Dean et al., 2013; Kirkwood and Betty, 2003).**

RESULTS

There was a non-significant difference between the two control groups all over PM I regarding the biochemical and histopathological investigation.

I- Biochemical results

1- pH:

There was a significant decrease in the PH value with the increase in PMI; however, this decrease was non-significant between 4-12 h PM in all groups and between 12-24 h in the Mesterolone group only. On the other hand, the Mesterolone group showed significantly higher PH values than the control groups at zero, 12 & 24 h PM, while no significant difference was detected at 4 h PM **(Table1).**

2- Oxidative stress markers:

Glutathione (GSH)

All 3 groups showed a significant decrease in GSH value with increasing in PMI starting from zero to 24 h PM. But the mesterolone groups were significantly lower than the control groups at all PM time points except at zero h the decrease was insignificant **(Table 2)**.

Malondialdehyde (MDA)

Myocardium showed a significant increase in MDA values with increasing PMI in all rats all over the four PM time points. On the other hand, there was no significant difference between the two control groups but mesterolone groups showed significantly higher MDA values than control groups at PM time points (4, 12, and 24 h) **(Table 3)**.

3- Adenotriphosphate (ATP) and Adenodiphosphate (ADP)

Myocardium in all groups showed a significant decrease in both ATP and ADP values at different PM time intervals starting from zero to 24 h. Conversely, ATP and ADP values of the mesterolone group were constantly significantly higher than control groups **(Tables 4, 5)**.

4- Glycogen

Heart muscles showed a significant decrease in glycogen levels with the increase in PMI, noting that the decrease was insignificant between 12-24 h PM in control groups. But glycogen levels of the mesterolone group were significantly higher than the control groups at all PM time points **(Table 6)**.

5- Lactic Acid

Heart muscles of all groups showed a significant increase in lactic acid concentrations with increasing PMI, but this increase was insignificant between 4-12 h PM in control groups' myocardium. Though the mesterolone group showed a significant decrease in its values compared to the control groups **(Table 7)**.

Histopathological Results:

1- Hematoxylin and Eosin Stain (H & E)

Specimens of the heart showed normal histological structures of striated, branched cardiomyocytes withacidophilic sarcoplasm and central oval vesicular nuclei. Intercalated discs appeared between cardiomyocytes. Flat dark nuclei of the fibroblasts were seen in the narrow interstitial tissue spaces at zero time and 4 h after death of all taken groups **(Fig.1 A, B, E, F)**. While, Specimens at 12 h and 24 h after death exhibited myocardial hyaline degenerations with more acidophilic sarcoplasm, and mild interstitial edema with round cell infiltrates which increased at 24 h after death, especially in the control group **(Fig.1 C,D,G,H)**. The cardiac muscle bundles were larger in diameter in the mesterolone groups than in control groups.

2- Masson Trichrome stain

Heart muscles stained by Masson's Trichrome showed collagen fibers at interstitial tissue which stained blue and declined in all groups from 0 h to 24 h after death **(Fig.2A-D & E-H)**. Noting that there is less stained collagen in interstitial tissue in control groups **(Fig.2 A-D)** than mesterolone -treated group **(Fig.2 E-H)**. The cardiac muscle fibers were larger in diameter in mesterolone-treated animals than in control groups.

II- Morphometric results

The mean area percent of the collagen fibers deposition in cardiac muscle sections gradually decreases with the increase of PMI. Yet, there was a significant increase in collagen fibers deposition in mesterolone -treated group compared to the control groups and there was a non-significant difference between the two control groups **(Table 8)**.

Table (1): PH values in heart tissue of (control water, control oil, and mesterolone) groups at different (zero, 4, 12, and 24 h) postmortem time points.

		Mean	SD	F	P
PH_M	Control water Group	7.2941	.01186	587.872	0.00**
	Control corn oil Group	7.3003	.03332		
	Mesterolone Group	7.7707*	.11765		
PH_M_4H	Control water Group	6.8146	.03136	1.436	0.242
	Control corn oil Group	6.8215	.04394		
	Mesterolone Group	7.0905	.13818		
PH_M_12H	Control water Group	6.5328	.03316	44.733	0.00**
	Control corn oil Group	6.5325	.02734		
	Mesterolone Group	6.7983*	.13876		
PH_M_24H	Control water Group	6.2128	.03316	10.895	0.00**
	Control corn oil Group	6.2202	.03534		
	Mesterolone Group	6.5639*	.08910		
		P			
Control	Zero -4H	0.001**			
	Zero -12H	0.0005**			
	Zero -24H	0.00**			
	4H- 12H	0.1594			
	4H- 24H	0.003*			
	12H- 24H	0.015*			
Control corn oil Group	Zero -4H	0.009*			
	Zero -12H	0.006*			
	Zero -24H	0.002*			
	4H- 12H	0.197			
	4H- 24H	0.0017*			
	12H- 24H	0.014*			
Mesterolone Group Control	Zero -4H	0.034*			
	Zero -12H	0.044*			
	Zero -24H	0.008*			
	4H- 12H	0.0712			
	4H- 24H	0.041*			
	12H- 24H	0.0521			

Mean*= Group causes the significant difference P* = Significant (P< 0.05) P**= Highly significant (P< 0.01)

Table (2): Reduced Glutathione levels in heart tissue of (control water, control oil, and mesterolone) groups at different (zero, 4, 12, and 24 h) postmortem time points

		Mean	SD	F	P
GSH_M	Control water Group	2.5369	.19855	2.208	0.211
	Control corn oil Group	2.5207	.15144		
	Mesterolone Group	2.4180	.48064		
GSH_M_4H	Control water Group	2.2569	.19855	118.221	0.00**
	Control corn oil Group	2.2588	.18607		
	Mesterolone Group	1.7837*	.11202		
GSH_M_12H	Control water Group	2.0254	.09939	26.312	0.00**
	Control corn oil Group	2.0103	.18896		
	Mesterolone Group	1.4063*	.17933		
GSH_M_24H	Control water Group	1.8523	.09882	51.728	0.00**
	Control corn oil Group	1.8230	.09969		
	Mesterolone Group	1.3112*	.20360		
		P			
Control	Zero -4H	0.00**			
	Zero -12H	0.00**			
	Zero -24H	0.00**			
	4H- 12H	0.00**			
	4H- 24H	0.009*			
	12H- 24H	0.007*			
Control corn oil Group	Zero -4H	0.00**			
	Zero -12H	0.00**			
	Zero -24H	0.00**			
	4H- 12H	0.00**			
	4H- 24H	0.011*			
	12H- 24H	0.007*			
Mesterolone Group	Zero -4H	0.00**			
	Zero -12H	0.00**			
	Zero -24H	0.00**			
	4H- 12H	0.005*			
	4H- 24H	0.034*			
	12H- 24H	0.042*			

Mean*= Group causes the significant difference P* = Significant (P< 0.05) P**= Highly significant (P< 0.01)

Table (3): Malondialdehyde levels in heart tissue of (control water, control oil, and mesterolone) groups at different (zero, 4, 12, and 24 h) postmortem time points.

		Mean	SD	F	P
MDA_M	Control water Group	112.9923	4.38685	2.896	0.285
	Control corn oil Group	112.8833	5.57664		
	Mesterolone Group	113.9263	6.85842		
MDA4_M	Control water Group	119.5821	2.39374	59.251	0.00**
	Control corn oil Group	120.2695	2.79081		
	Mesterolone Group	128.0123*	4.95409		
MDA12_M	Control water Group	127.6185	2.31544	27.525	0.00**
	Control corn oil Group	128.5323	1.92995		
	Mesterolone Group	135.9320*	3.62475		
MDA24_M	Control water Group	131.3652	6.84217	67.263	0.00**
	Control corn oil Group	132.2564	4.76681		
	Mesterolone Group	141.633*	1.52575		
		P			
Control	Zero -4H	0.00**			
	Zero -12H	0.00**			
	Zero -24H	0.00**			
	4H- 12H	0.00**			
	4H- 24H	0.007*			
	12H- 24H	0.003*			
Control corn oil Group	Zero -4H	0.00**			
	Zero -12H	0.00**			
	Zero -24H	0.00**			
	4H- 12H	0.00**			
	4H- 24H	0.007*			
	12H- 24H	0.012*			
Mesterolone Group	Zero -4H	0.00**			
	Zero -12H	0.00**			
	Zero -24H	0.00**			
	4H- 12H	0.009*			
	4H- 24H	0.001**			
	12H- 24H	0.003*			

Mean*= Group causes the significant difference P* = Significant (P< 0.05) P**= Highly significant (P< 0.01)

Table (4): Adenosine Triphosphate values in heart tissue of (control water, control oil, and mesterolone) groups at different (zero, 4, 12, and 24 h) postmortem time points (ANOVA & paired by paired t tests).

		Mean	SD	F	P
ATP_M	Control water Group	30.1000	.99480	381.299	0.00**
	Control corn oil Group	30.0115	.86046		
	Mesterolone Group	47.3415*	6.83754		
ATP_M_4H	Control water Group	22.9590	2.06454	119.452	0.00**
	Control corn oil Group	22.4618	2.35720		
	Mesterolone Group	39.3207*	3.87870		
ATP_M_12H	Control water Group	17.5044	2.67458	396.586	0.00**
	Control corn oil Group	17.6060	2.49096		
	Mesterolone Group	33.7783*	1.88829		
ATP_M_24H	Control water Group	14.0731	3.23007	29.361	0.00**
	Control corn oil Group	13.9693	3.36426		
	Mesterolone Group	29.1180*	5.18562		
		P			
Control	Zero -4H	0.00**			
	Zero -12H	0.00**			
	Zero -24H	0.00**			
	4H- 12H	0.00**			
	4H- 24H	0.00**			
	12H- 24H	0.00**			
Control corn oil Group	Zero -4H	0.00**			
	Zero -12H	0.00**			
	Zero -24H	0.00**			
	4H- 12H	0.00**			
	4H- 24H	0.00**			
	12H- 24H	0.00**			
Mesterolone Group	Zero -4H	0.00**			
	Zero -12H	0.00**			
	Zero -24H	0.00**			
	4H- 12H	0.003*			
	4H- 24H	0.001**			
	12H- 24H	0.009*			

Mean*= Group causes the significant difference P* = Significant (P< 0.05) P**= Highly significant (P< 0.01)

Table (5): Adenosine Diphosphate values in heart tissue of (control water, control oil, and mesterolone) groups at different (zero, 4, 12, and 24 h) postmortem time points (ANOVA & paired by paired t tests).

		Mean	SD	F	P
ADP_M	Control water Group	5.4269	.20613	44.021	0.00**
	Control corn oil Group	5.3585	.05890		
	Mesterolone Group	6.6127*	.12444		
ADP_M_4H	Control water Group	4.6479	.20579	76.688	0.00**
	Control corn oil Group	4.5792	.06091		
	Mesterolone Group	6.4734*	.13169		
ADP_M_12H	Control water Group	3.9446	.20781	38.538	0.00**
	Control corn oil Group	3.9743	.07078		
	Mesterolone Group	5.1829*	.16196		
ADP_M_24H	Control water Group	3.3592	.21530	11.538	0.00**
	Control corn oil Group	3.3883	.09915		
	Mesterolone Group	4.5837*	.57490		
		P			
Control	Zero -4H	0.00**			
	Zero -12H	0.00**			
	Zero -24H	0.00**			
	4H- 12H	0.00**			
	4H- 24H	0.008*			
	12H- 24H	0.006*			
Control corn oil Group	Zero -4H	0.00**			
	Zero -12H	0.00**			
	Zero -24H	0.00**			
	4H- 12H	0.00**			
	4H- 24H	0.007*			
	12H- 24H	0.009*			
Mesterolone Group	Zero -4H	0.00**			
	Zero -12H	0.00**			
	Zero -24H	0.00**			
	4H- 12H	0.007*			
	4H- 24H	0.001**			
	12H- 24H	0.004*			

Mean*= Group causes the significant difference P* = Significant (P< 0.05) P**= Highly significant (P< 0.01)

Table (6): Glycogen content in heart tissue of (control water, control oil, and mesterolone) groups at different (zero, 4, 12, and 24 h) postmortem time points.

		Mean	SD	F	P
Glycogen M	Control water Group	7.0200	.00946	3386.962	0.00**
	Control corn oil Group	7.0230	.00939		
	Mesterolone Group	8.7295*	.18393		
Glycogen_M_4H	Control water Group	5.0664	.13600	326.066	0.00**
	Control corn oil Group	5.0560	.12062		
	Mesterolone Group	6.5861*	.09977		
Glycogen_M_12H	Control water Group	4.5833	.18365	214.632	0.00**
	Control corn oil Group	4.5675	.11309		
	Mesterolone Group	6.0227*	.18138		
Glycogen_M_24H	Control water Group	4.3162	.19088	9.0528	0.00**
	Control corn oil Group	4.2657	.14377		
	Mesterolone Group	5.8415*	.20150		
		P			
Control	Zero -4H	0.00**			
	Zero -12H	0.00**			
	Zero -24H	0.00**			
	4H- 12H	0.02*			
	4H- 24H	0.001**			
	12H- 24H	0.211			
Control corn oil Group	Zero -4H	0.00**			
	Zero -12H	0.00**			
	Zero -24H	0.00**			
	4H- 12H	0.021*			
	4H- 24H	0.001**			
	12H- 24H	0.198			
Mesterolone Group	Zero -4H	0.00**			
	Zero -12H	0.00**			
	Zero -24H	0.00**			
	4H- 12H	0.00**			
	4H- 24H	0.00**			
	12H- 24H	0.00**			

Mean*= Group causes the significant difference P* = Significant (P< 0.05) P**= Highly significant (P< 0.01)

Table (7): Lactic acid concentrations in heart tissue of (control water, control oil, and mesterolone) groups at different (zero, 4, 12, and 24 h) postmortem time points.

		Mean	SD	F	P
Lactic acid_M	Control water Group	20.2312	2.1452	7.965	0.00**
	Control corn oil Group	20.0521	1.8564		
	Mesterolone Group	16.254*	2.6321		
Lacti acid_M_4	Control water Group	25.5679	1.9854	34.094	0.00**
	Control corn oil Group	25.6810	1.8541		
	Mesterolone Group	20.8754*	1.7452		
Lactic acid_M_12	Control water Group	27.8251	2.5541	4.626	0.002*
	Control corn oil Group	27.9521	1.9656		
	Mesterolone Group	24.632*	2.214		
Lactic acid_M_24	Control water Group	31.3949	1.20419	6.375	0.002*
	Control corn oil Group	31.9400	1.30628		
	Mesterolone Group	27.8502*	1.05725		
		P			
Control	Zero -4H	0.003*			
	Zero -12H	0.006*			
	Zero -24H	0.002*			
	4H- 12H	0.122			
	4H- 24H	0.028*			
	12H- 24H	0.034*			
Control corn oil Group	Zero -4H	0.007*			
	Zero -12H	0.003*			
	Zero -24H	0.001**			
	4H- 12H	0.085			
	4H- 24H	0. 009*			
	12H- 24H	0.014*			
Mesterolone Group	Zero -4H	0.033*			
	Zero -12H	0.038*			
	Zero -24H	0.007*			
	4H- 12H	0.0412*			
	4H- 24H	0.002*			
	12H- 24H	0.0255*			

Mean*= Group causes the significant difference P* = Significant (P< 0.05) P**= Highly significant (P< 0.01)

Table (8): Statistical comparison of the area percent of collagen fibers in heart sections stained by Masson Trichrome stain among groups (control water, control oil, and Mesterolone) at different (zero, 4, 12, and 24 h) postmortem time points

		Mean	SD	F	P
Zero-h	Control water Group	0.94	0.08	7.0544	0.0266*
	Control corn oil Group	0.95	0.06		
	Mesterolone Group	1.58*	0.4		
4 h	Control water Group	0.92	0.07	21.0745	0.0019**
	Control corn oil Group	0.93	0.08		
	Mesterolone Group	1.37*	0.13		
12 h	Control water Group	0.9	0.06	5.3632	0.046*
	Control corn oil Group	0.91	0.07		
	Mesterolone Group	1.26*	0.25		
24 h	Control water Group	0.87	0.09	12.7052	0.007**
	Control corn oil Group	0.88	0.045		
	Mesterolone Group	1.17*	0.1		

Mean*= Group causes the significant difference P* = Significant (P< 0.05) P**= Highly significant (P< 0.01)

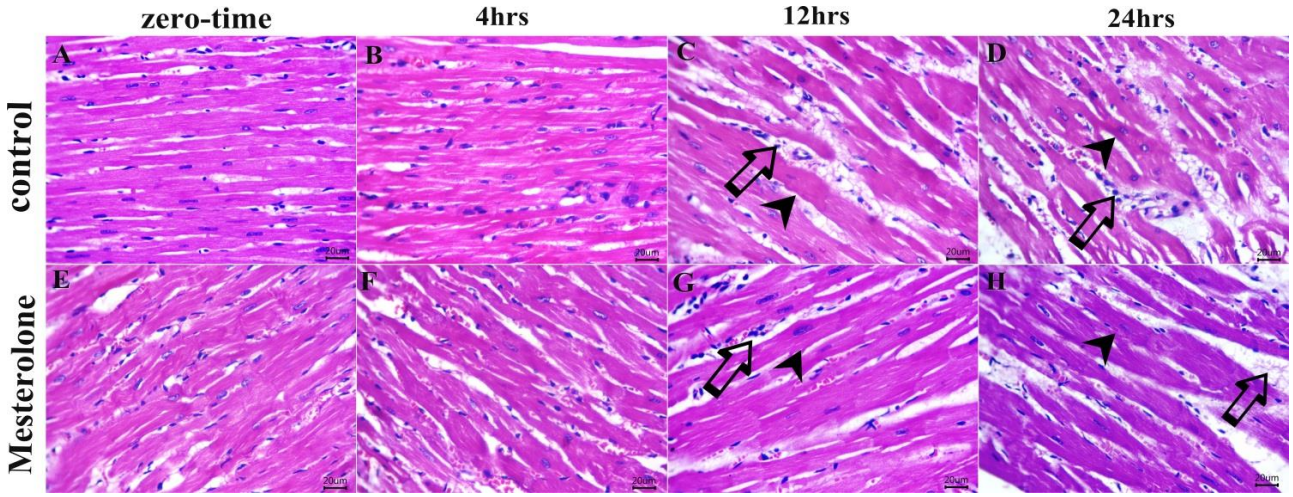

Fig.(1): *A photomicrograph of H&E-stained sections in the heart muscle of adult male albino rats showing normal histological structures of striated, branched cardiomyocytes in specimens at zero-time and 4 h after the death of control* ***(A and B)*** *and mesterolone-treated animals* ***(E and F).*** *In 12 and 24 h after death, there are myocardial hyaline degenerations* ***(arrowheads)****, mild interstitial edema with round cell infiltrates* ***(arrow)*** *(C, D, G, H) with increasing edema and inflammatory cells at 24 h after deaths in the control groups (D).* ***Scale bar 20 µm.***

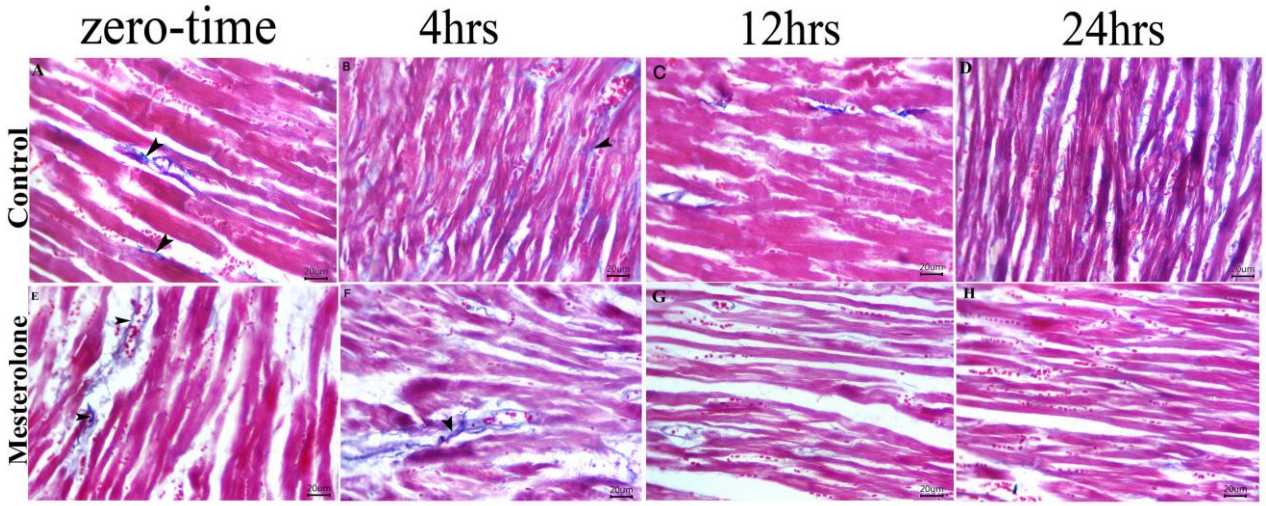

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Fig. (2): *A photomicrograph of Masson's Trichrome stained sections in the heart muscle of adult male albino rats showing blue staining collagen fibers at interstitial tissue at zero time, 4 h, 12 h, and 24 h after death in the control groups* ***(A, B, C, and D)****, with more collagen fibers in heart muscles* ***(arrowhead)*** *of mesterolone -treated animals* ***(E, F, G, and H)****. There is a gradual decrease in collagen deposition within the interstitial tissue from 0 to 24 h after death in control groups* ***(A-D)*** *and mesterolone-treated group* ***(E-H). Scale bar (20 µm).***

DISCUSSION

Several studies have identified fatal cases linked to long-term steroid abuse. The autopsies of these deceased often revealed pathological changes of the myocardium, such as cardiac and left ventricular hypertrophy, coronary atherosclerosis, myocarditis, interstitial and replacement fibrosis, and contraction band necrosis. In such cases, cardiac arrest is often described as the cause of death ***(Lehmann et al., 2019).*** It is hard to distinguish the etiology of these changes from histological findings alone. Consequently, ***Kanayama et al. (2020)*** recommended evaluating the subject's clinical history and physical characteristics in all cases of sudden cardiac death in which AAS abuse is suspected.

Moreover, ***Frati et al. (2015)*** documented that AASs users frequently consume numerous types, in different forms, singularly and in several temporal combinations and cycles, and commonly, various steroid-accessory drugs are also used. Therefore, interpreting the postmortem findings in AAS-related deaths is particularly difficult, and no comprehensive conclusions can be performed. The numerous studies in animal models,

especially in rats, have called into question several pathophysiological mechanisms, which may explain various macroscopic and microscopic findings regarding the AASs related death.

In the current study, mesterolone was orally administered at a high therapeutic dose that mimics the mesterolone misuse and abuse by bodybuilders. Biochemical and histological changes were investigated in the cardiac muscle of rats after twelve weeks of oral exposure to mesterolone at different PMI.

The present study recorded a significant decrease in the heart tissue pH values with increasing PMI. A drop in pH value proportional to PMI was previously reported by ***Donaldson and Lamont (2013)*** *and* ***Brooks (2009).***

Brooks (2018) explained that the PM drop in pH value in tissues could be due to increased lactic acid production resulting from PM anaerobic glycolysis.

Mesterolone administration significantly caused higher pH values of heart muscles than control at zero, 12 and 24 h PM**.**

Elmajdoub et al., (2016) observed that anabolic steroid treatment significantly elevated pH relative to the control group. Congruently, breast muscle's pH value was increased considerably in bolderon (BOL)-treated broiler chicks.

The raised pH values in heart tissue could be attributed to the fall in lactic acid concentrations in mesterolone-treated rats compared to the control group in the current study.

In the present study, reduced glutathione levels decreased significantly in the heart muscle of all groups all over PM time points. On the other hand, malondialdehyde levels increased at all PMI.

In accordance with the present study, ***El-Noor et al. (2016)*** investigated the changes of oxidative stress markers in the heart and kidney after death; and proved significant positive correlations to MDA and NO and negative correlations to glutathione reductase (GR), glutathione S transferase, and catalase.

Under physiological conditions, there is a continuous balance between oxidants and antioxidant defense systems in favor of antioxidants. Meanwhile, in injured tissue, this equilibrium favors oxidants with a decline in the antioxidant activity in any live animal. After death, endogenous oxidant/antioxidant equilibrium may be inadequately controlled by the body, so alterations in oxidant/antioxidant status can be assessed as postmortem biochemical perturbation ***(Mostafa et al., 2021).*** This could explain the significant and progressive reduction in GSH activity and parallel elevation of MDA levels with increasing postmortem intervals in the present study.

In the same context, ***Harish et al. (2011)*** found that malondialdehyde started to increase significantly at 4-5 hours in brain tissue and at 3-4 hours in the liver tissue after death. The increased MDA levels with increasing PMI could be attributed to increased lipid peroxidation. This subsequently increased binding and detoxification by GSH, which eventually reduced.

The interaction between PMI and mesterolone treatment induced more oxidative damage in cardiac tissues indicated by a decrease in the activity of the antioxidant enzyme (GSH) together with an increase in lipid peroxidation (MDA) in mesterolone-treated rats.

The present data coincided with those in the former report demonstrating that AASs encouraged oxidative burden in rabbits' tissues ***(Ali et al., 2013)***. Also, ***Mayada et al. (2015) and Behairy et al. (2020)*** discussed that AASs could increase ROS levels and oxidative stress biomarkers in various organs such as the liver and testis.

This study showed significantly higher ATP and ADP values in the cardiac muscles of mesterolone-treated rats compared to the control groups at all PM time points.

Similarly, ***Elmajdoub et al. (2016)*** reported that the bolderon administration significantly elevated the concentrations of ATP and ADP compared to the control group. These findings indicated that the anabolic steroids treatment could induce a significant boost of energy production in the muscles.

Anabolic androgenic steroid administration boosts erythropoiesis. Therefore, the increase in the number of circulating red blood cells contributes to the rise in the body's capacity to supply tissues with oxygen, which is used

for energy production and increasing ATP levels via oxidative phosphorylation ***(Jelkmann et al., 2009).***

On the other hand, the present study recorded a significant decrease in ATP and ADP values in the heart muscles of all groups with increasing PMI starting from zero to 24 h.

In line with these findings**,** ***Huang et al. (2010)*** recorded a significant PM-time-dependent reduction in the ATP and ADP levels in the gastrocnemius muscle of rats.

Logotheti et al. (2018) explained that ATP and ADP values significantly reduced after death because the oxygen supply to tissues is stopped. Postmortem anoxia would lead to the cessation of glycolysis and phosphocreatine depletion. ATP synthesis shortly stopped along with a rapid breakdown of ATP to ADP, then adenosine monophosphate (AMP) and inosine monophosphate (IMP) by simple enzymatic reactions.

In this context, a previous study by ***Zhu et al. (2021)*** demonstrated that ATP assessment and its degradation products could be possible biomarkers in PMI determination.

In the current study, the mesterolone-treated group showed significantly higher glycogen values in heart muscles compared to control groups at all PM time points. While lactic acid levels significantly decreased compared to control groups.

In line with the present results, ***Saber et al. (2021)*** demonstrated a significant elevation in the glycogen level concurrent with a decrease in the lactic acid in the muscle of the bolderon-treated group compared to the control one.

The results of the current study could be supported by ***Carvalho et al. (2020)***, who reported a significant increase in the glycogen content and a substantial decrease in the lactic acid in the rat liver after bolderon-exposure. This boost in glycogen stores can be explained by AAS- induced down-regulation of glycogen phosphorylase enzyme and up-regulation of glycogen synthetase I activity.

On the contrary, the heart muscles of all the experimental groups showed a significant decrease in glycogen accompanied by increased lactic acid values in a PM time-dependent manner.

The PM glycogen depletion could be related to postmortem glycogen degradation via anaerobic glycolysis due to insufficient tissue oxygen supply after death, which in turn increases the lactic acid concentration ***(Powers, 2005).***

Heart showed preserved histological arrangements in specimens taken from all the study groups at zero-time and 4 h after death. At 12 and 24 h after death, myocardium exhibited hyaline degeneration with mild interstitial edema and round cell infiltrates that increased with increasing PM time, especially in the control group. Cardiac muscle bundles were larger in diameter in the mesterolone group compared to the control.

In line with the present results**,** ***El-Noor et al. (2016)*** revealed gradual deterioration in the histopathological structure, which matched in time with the increase in oxidant levels and decrease in antioxidant levels. They described almost normal myocardial muscle of the heart during the first-hour postmortem; at 3 to 4 h PM myocardium were wavy bundles with widening of interstitial spaces; at 4 to 5 h PM, swollen myocardium displayed dislodgement of the nuclei, loss of striations, and eosinophilic cytoplasm; at 5 to 6 h PM heart was changed into a continuous sheet of highly eosinophilic structure and lysis of myocardial fibers; at 6 to 7 h PM heart showed extensive autolysis with disruption of cellular structure and wide splitting of the myofibrils.

Tomita et al. (1999) suggested that histological alterations can be used as markers for PMI estimation.

Postmortem hypoxia leads to ATP loss, leading to the stoppage of various energy-dependent cellular systems such as ion pumps (resulting in cell puffiness and Ca2+influx) and glycogen depletion, with the build-up of lactic acid and lowering the cellular pH. These circumstances diminish protein synthesis along with degradation and atrophic changes. The continuous hypoxia worsens ATP depletion and advocates free radical accumulation, which causes cell membrane damage by cross-linking proteins and lipid peroxidation of cellular membranes ***(Mostafa et al., 2021)***. This could explain the progressive histological deterioration that occurred in heart tissue in this study.

In the current study, mesterolone treatment induced an enlargement in the diameter of cardiac muscle bundles compared to the control group. In this regard, an earlier report by ***Mohamed and Mohamed (2022)*** verified the relationship between AAS and abnormalities in muscle morphology. They declared that the steroid-treated animals showed apparent hypertrophy of muscle fibers and disrupted striations with wide spacing between them.

In the present study, heart muscle sections stained with Masson's Trichrome showed blue stained collagen fibers at interstitial tissue that decreased gradually from 0 h to 24 h after death. There was more stained collagen in the interstitial tissue of mesterolone -treated group than in the control. Also, cardiac muscle fibers were larger in diameter in mesterolone-treated animals compared to control groups.

Numerous studies have used Masson's trichrome stain to identify the collagen fibers regardless of collagen type ***(Chen et al., 2017).*** Masson's trichrome staining is widely implemented in the study of cardiac pathologies. Many previous researchers used Masson's trichrome stain to determine the amount of collagen deposition in tissues and their orientation in different stages of their products, especially in the wound healing and fibrosis process ***(Al-Mahmood, 2020).***

The present data indicated the occurrence of collagen degradation after death. Numerous studies have reported postmortem collagen degradation in *tissues* ***(Jellinghaus et al., 2019; Mills et al., 1989).*** Controversy, the mesterolone treatment significantly increased the collagen fibers reaction in cardiac muscles compared to that in control rats. This effect could be related to the stimulation of collagen synthesis by AAS ***(Falanga et al., 1998; Pärssinen et al., 2000).***

In line with the present findings***, Hanan et al. (2018)*** observed a widening of the interstitial tissue in the cardiac muscles of rats treated with AAS. They discussed that could be the result of a progressive increase in the connective tissue elements, especially collagen fibers, and was concomitant with the degree of muscle damage. Loss of the contractile ability of the heart could be attributed to the collagen associated with cardiac hypertrophy and increased stiffness or decreased compliance of the ventricular wall that may give rise to heart failure ***(Franquni et al., 2013).***

Also, the results of the present study were consistent with ***El-Naeem et al. (2022)***, who reported that Masson's trichrome staining could detect a different degree of cardiac fibrosis. The observed more collagen fibers could be explained by the increased free-radical resulting in the induction of more fibrosis in cardiac muscles.

Animal studies are imperative as they confirm that AASs act directly on heart cells by changing conformation and causing direct damage that can lead to sudden cardiac death. For instance, studies on isolated hearts from rats exposed to chronic nandrolone decanoate (ND) have shown a rise in myocardial susceptibility to ischemia-reperfusion injuries. Also, several researchers studied the effects of AAs on cardiac function in rats undergoing swimming training. They found that swimming training combined with high doses of nandrolone aggravates cardiac hypertrophy with interstitial fibrosis ***(Wadthaisong et al., 2019).***

From the results of the current study, we can conclude that chronic use of mesterolone has led to marked biochemical and histological changes in the heart. Also, mesterolone had notable modifying effects on the energy content, oxidant/antioxidant parameters, and histological architecture of the heart in the first 24 h PM. Hence, they can be used as an indicator for the determination of early PMI. These results are preliminary, although significant results were found in this animal study. Therefore, it can be recommended that further research on animals for more duration, different PMI, and others on humans is necessary. Forensic examiners should consider mesterolone treatment's interactions with PM changes when performing postmortem investigations in athletes, AAS-treated patients, and cases of sudden death in young adults.

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آثار التناول المزمن للميسترولون على تغيرات ما بعد الوفاة في قلب ذكور الجرذان البيضاء البالغة (دراسة بيوكيميائيه وهيستوباثولوجية)

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الملخص العربى

المقدمة: ان استخدام المنشطات الاندروجينيه الابتنائيه من قبل الرياضيين المحترفين في ازدياد حول العالم.

الهدف من العمل: هو دراسه تغيرات ما بعد الوفاة في غضون الـ 24 ساعة الأولى في قلب الفئران المعالجة بالميسترولون.

المواد والطرق: تم تقسيم مائه وعشرون جرذا من ذكور الجرذان البالغة إلى مجموعتين. المجموعة الأولى (المجموعة الضابطة): تضم ثمانون جرذًا ، مقسمة إلى مجموعتين فرعيتين متساويتين.

المجموعة الثانية (الميسترولون) (اربعون جرذا): عولجت بـ 2.14 ملجم / كجم من وزن الجسم من الميسترولون مذاب في 1 مل من زيت الذرة.

تم اعطاء الجرعات عن طريق الفم مرة واحدة يوميا لمدة اثني عشر أسبوعا. تم ذبح الفئران وجمع عينات القلب (البطينين) في وقت الصفر ، 4 ، 12 ، 24 ساعة بعد الوفاة لاستخدامها في الفحوصات البيوكيميائية والهستوباثولوجية.

النتائج: أدى استخدام الميسترولون إلى زيادة معنوية ذات دلالة احصائية في قيم الأس الهيدروجيني والأدينوزين ثلاثي وثنائي الفوسفات والمالندهيد والجليكوجين وكذلك خفض مستويات الجلوتاثيون وحمض اللاكتيك في عضلة القلب عند مقارنته بالمجموعات الضابطة.

كما حدث انخفاض في قيم الأس الهيدروجينيومضادات الأكسدة ، والأدينوزين ثلاثي وثنائي الفوسفات ، ومستويات الجليكوجين وارتفاع في مستويات اكسدة الدهون وحمض اللاكتيك بشكل ملحوظ بالتوازي مع زيادة الوقت بعد الوفاة في عضلات القلب في كل من المجموعتين الضابطة والميسترولون.

تم الكشف عن تدهور تدريجي في الأنسجة العضلية والكولاجين متزامن مع مرور الوقت في فترة ما بعد الوفاة في حين يزيد الميسترولون بشكل ملحوظ من تكوين ألياف الكولاجين في عضلات القلب.

الخلاصة: يجب مراعاة النتائج السابقة عند تحديد فترة ما بعد الوفاة في الحالات التي لها تاريخ من إساءة استخدام المنشطات الاندروجينيه الابتنائية أو المرضى الذين عولجوا مسبقًا بهذه الأدوية لفترات طويلة أو في حالات الموت المفاجئ عند البالغين الأصحاء حيث أن تناول المسترولون يؤثر على محتوى الطاقة وحالة الأكسدة والبنية النسيجية لعضلات القلب في الفترة المبكرة بعد الوفاة.