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Review Article

ULTRASONOGRAPHIC AND HISTOPATHOLOGIC STUDY OF CHEMICAL CASTRATION WITH CALCIUM CHLORIDE SOLUTION IN RAT TESTIS

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ABSTRACT

Chemical castration is a method of castration by drug. In this way, unlike sterilization of sex glands those are removed through surgery, Glands are undermined by injecting chemicals such as calcium chloride into the tissue. The aim of this study was to assess the effects of calcium chloride solution for injection in rat testis tissue with ultrasonography and histopathology, in analyzing the testis. 20 male Wistar Rats with an average age of 6 months and weighing approximately 250-200 g, clinically healthy, were selected. The mice were then randomly divided into 4 equal groups. The first group was considered as a control group. Then, in the other three groups, after the measurements, 20% calcium chloride was injected into the testicular tissue, so that its tissue was completely filled. Then, on the 7th, 14th and 21st days, samples were taken from the right testicles of the groups for histopathological examination and were examined by hematoxylin and eosin staining. The length, width and echogenicity of all testes on Zero, 7th 14th and 21st days were also examined by ultrasonography. Finally, the results of quantitative and semi-quantitative study of the measured components were statistically analyzed. The results of ultrasonography showed that firstly the injection length and width between the left and right testis there is no significant difference between groups. But on 7th, 14th and 21st days significant difference between them is seen. Secondly, the average length and width of the left and right testicles in both groups during the study (7th, 14th and 21 days after injection) significantly decreased. Thirdly Echogenicity of left and right testis in groups during the experiment significantly reduced. In addition, histopathological examination revealed that thein the testicular tissue of the experimental groups, cellular necrosis, hemorrhage, neutrophilia and calcification was seen. Conclusion: Based on the findings of this study, it can be concluded that chemical sterilization using calcium chloride is an effective method and reduces costs, reduces side effects and eliminates anesthesia. But the effects of these methods appear slowly.

Keywords: Ultrasonography, Histopathology, Testis, Calcium chloride, Wistar Rat.

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INTRODUCTION

Sterilization is one of the effective ways to control animal populations. Today researchers consider chemical castration as an alternative to surgery. Because this method has many advantages compared to surgical procedures, such as reducing stress, relieve bleeding, hernia, infection, Mysis, the need for sterile environment which is. Simplicity and cheapness, especially in mass scale, are other advantages of this method. [1]

Castration can be used with various chemicals. The most common Materials are: cadmium chloride,[2] metallic boron, dexamethasone, methoprim, niridazole, alpha chlorohydrin[3], ferric chloride and ferrous sulfate[4], glycerol[5], lactic acid and calcium chloride[6]. These substances affect spermatogenesis, endogenesis, testicular atrophy, and epididymis and stop sexual desire[7].

Injection of testicular calcium chloride concentration and different formulas, to a variety of animals, such as rats [8,9], dogs [10-12], cats [13], goats [14,15], and bulls [1,16,17], have been reported. According to reports published in the calcium chloride can result in atrophy of the seminiferous tubules and reduce circulating levels of testosterone and sperm count and testicular atrophy in male animals well be [18].

But despite the numerous benefits listed for this method, in some reports, local and systemic reactions, such as scrotal ulcers and dermatitis, and abscess and fibrosis in the area, peripheral edema, vomiting, diarrhea, anorexia, lethargy, and leukocytosis have been reported [19-22]. Also, some researchers believe that castration by chemical methods does not reduce the gonadal sources of testosterone [21].

In any event, the aim of this study was to investigate the changes in testicular tissue due to injection of Calcium Chloride into tissue using two methods: ultrasonography and histopathology.

MATERIALS AND METHODS

Animals: Animals used in this study included 20 male Wistar rats with an average age of 6 months and weighing approximately 250-200 g.
All mice before and after the start of the study (21 day) complete clinical examination (including Temperature check, testicular pain, appetite and stool) were ensured the safety of all of them.

Groups: Animals were randomly divided into 4 equal groups. Each of the group are:
Group I: control
Group II: The second group was injected with Calcium Chloride solution in a zero-day and withdrawal right testicle on 7th day
Group III: injection of Calcium Chloride solution in a zero-day and withdrawal right testicle on 14th day
Group IV: injection of Calcium Chloride solution in a zero-day and withdrawal right testis on 21st day

Injection: The first was a short-haired hairs in the testes of mice with the modified device. The topical lidocaine to numb the area. Then a solution of calcium chloride 20% (now Company) under the guidance of ultrasound to the testes were injected. For this purpose, half the length of the sterile syringe with a gauge of 27 inches from the ventral surface of the testicles, far from the epididymis, was inserted into the cranial testicle. The injection was performed slowly so that the pressure caused by the filling of this solution was felt inside the testicular tissue under the scrotum. The injection volume was determined after a pilot test, so that the injection volume was released in the testes, but did not leak out from the testicles.

Ultrasonography: To investigate the effect of Calcium Chloride solution on testes, the length, width and echogenicity of both testes were measured before the chemical injection and on 7th, 14th and 21st days after the injection. For this purpose, the rats were placed behind and with a frequency of 7.5 MHz and Micro-convex probe and Mindray Ultrasonographic Device, The Necessary investigations were carried out.

It should be noted that in order to examine the testicular echogenicity were assigned, low brightness 1, medium brightness 2, high brightness 3 and very high brightness 4.

Also, to reduce measurement errors, the entire process was carried out by three radiologists.

Histopathology: Tissue To investigate the testicular tissue, the left testicles of the experimental groups were fixed in 10% Buffered Formalin on the 7th , 14th and 21st day and transferred to the pathology department of Islamic Azad University, Science and Research Branch. Tissue formalin was also replaced 24 hours later and remained in place until studies were completed. And then placed in paraffin blocks. It was then examined microscopically by hematoxylin and eosin staining. For this purpose, the following 5 factors were measured:

1. Intravascular thrombosis
2. Seminiferous Tubule necrosis
3. Bleeding
4. Interstitial Neutrophilic infiltration
5. Calcification

The damage observed was measured according to the following quantitative indicators:

0: lack of damage
1: the least damage
2: average damage
3: a large amount of damage
4: too much damage

Statistical Analysis: Data were analyzed with version 21 SPSS software, using ANOVA test (for comparison of multiple means) and dependent t-test (to compare the mean of each group in several stages of the experiment) and taking into account the values of P <0.05 for significant Perceptions of differences were statistically analyzed.

RESULTS

a. Ultrasonography:

The results of quantitative and qualitative examination of the testes following injection of sodium chloride solution are reported separately in the following lines.

1. The results of comparing the length and width of the left and right testicle between the groups

The results of one-way ANOVA test between control groups, first, second and third in the components of length and width of the left testicle and the length and width of the right testicle at different times are as indicated in the following table.

Table 1. Results of ANOVA analysis test to compare the mean of the tested groups in the length and width components in the left and right testes

Time	Testicles		Sum of squares	Df	F	Sig
Before injection	Left testicle	Length	8.23	3	2.39	**0.10**
		Width	9.69	3	0.88	**0.47**
	Right testicle	Length	6.56	3	3.09	**0.06**
		Width	13.71	3	0.64	**0.59**
7th day	Left testicle	Length	8.05	3	6.39	**0.005**
		Width	6.86	3	13.05	**0.00**
	Right testicle	Length	12.41	3	22.07	**0.00**
		Width	8.91	3	31.07	**0.00**
14th day	Left testicle	Length	6.57	3	127.43	**0.00**
		Width	1.95	3	373.38	**0.00**
	Right testicle	Length	3.19	3	460.82	**0.00**
		Width	3.19	3	460.82	**0.00**
21st day	Left testicle	Length	7.80	3	157.15	**0.00**
		Width	2.53	3	415.65	**0.00**
	Right testicle	Length	1.08	3	1.78	**0.00**
		Width	1.64	3	1.04	**0.00**

The results showed that there was a statistically significant difference between the means of the groups. Tukey's multiple comparison post-hoc test was used to identify different means. The results showed that there was a statistically significant relationship between the mean of the control group, second and fourth on 7th, 14th and 21st days (P<0.05)

2. The results of comparing the length and width of the testicles in each group

The results of t-test to compare the length and width of the left and right testicles in the second, third and fourth groups on the pre-injection days and on days 7, 14 and 21 are listed in Table 2:

Table 2. Comparing the length and width of the left and right testicles on pre-injection day, and 7th, 14th and 21 days in the group II, group III and group IV

Group	Time	Size	Left testicle				right testicle			
			T	df	Sig	mean	T	df	Sig	mean
Group II	Before injection	Length	41.80	4	0.00	11.34	22.48	4	0.00	**10.38**
		Width	18.54	4	0.00	9.40	16.73	4	0.00	**10.09**
	7th day	Length	21.77	4	0.00	9.38	15.59	4	0.00	**9.33**
		Width	26.13	4	0.00	7.92	18.82	4	0.00	**8.65**
	14th day	Length	17.38	4	0.00	3.45	0	0	0	**0**
		Width	17.48	4	0.00	3.25	0	0	0	**0**
	21st day	Length	8.26	4	0.00	2.46	0	0	0	**0**
		Width	14.79	4	0.00	2.26	0	0	0	**0**
Group III	Before injection	Length	25.35	4	0.00	11.70	41.64	4	0.00	**11.47**
		Width	38.74	4	0.00	9.35	47.37	4	0.00	**9.29**
	7th day	Length	32.89	4	0.00	9.04	31.54	4	0.00	**7.49**
		Width	20.65	4	0.00	7.68	31.55	4	0.00	**6.25**
	14th day	Length	12.05	4	0.00	4.65	13.61	4	0.00	**4.24**
		Width	70.25	4	0.00	4.13	13.61	4	0.00	**4.24**
	21st day	Length	13.41	4	0.00	2.77	0	0	0	**0**
		Width	14.92	4	0.00	2.60	0	0	0	**0**
Group IV	Before injection	Length	157.15	4	0.00	11.76	82.81	4	0.00	**10.81**
		Width	26.48	4	0.00	9.94	19.68	4	0.00	**9.70**
	7th day	Length	60.30	4	0.00	8.93	15.03	4	0.00	**6.29**
		Width	27.18	4	0.00	7.88	18.25	4	0.00	**6.29**
	14th day	Length	33.94	4	0.00	4.88	24.73	4	0.00	**4.53**
		Width	26.50	4	0.00	4.66	24.73	4	0.00	**4.53**
	21st day	Length	8.95	4	0.00	3.42	20.38	4	0.00	**3.24**
		Width	15.74	4	0.00	3.32	16.16	4	0.00	**3.31**

The information contained in Table 2 show that the mean length and width of the left and right testicles in the second group at different time's significant difference exists. So that the highest mean is related to pre-injection and the lowest mean is related to day 21. There is a decreasing trend in it.

Besides that, the data in Table 2 shows that the length and width of the left and right testicles decreased at the time of the study, ie before the chemical injection and day 7, day 14 and day 21 in the third group, and this decrease was statistically significant. Notes that the right testicle was surgically removed after measurement on day 14 for histopathological evaluation.

Furthermore in Table 2, there is a significant relationship between the length and width components of the left and right testicles in the fourth group before the injection and on days 7, 14 and 21 after the injection, and as a means of known the length and width of the study appeared declining.

3. The results of comparing the Echogenicity of the left and right testicle in each groups

The results of left and right testicular echogenicity in the second, third and fourth groups in the pre-injection day, 7th, 14th and 21st days are listed in the following table:

Table 3. Left and right testicular echogenicity on pre-injection day, and 7th, 14th and 21 days in the group II, group III and Group IV

Group	esticle	Time	Number	t	Df	Sig	Mean
group II	Left testicle	Before injection	5	12.64	4	0.00	4.00
		7th day	5	5.88	4	0.00	2.20
		14th day	5	9.00	4	0.01	1.80
		21st day	5	-	-	-	1.00
	Right testicle	Before injection	5	14.69	4	0.00	3.60
		7th day	5	11.00	4	0.00	2.20
		14th day	5	-	-	-	0
		21st day	5	-	-	-	0
Group III	Left testicle	Before injection	5	19.00	4	0.00	3.80
		7th day	5	5.89	4	0.00	2.20
		14th day	5	5.88	4	0.00	2.20
		21st day	5	5.71	4	0.05	1.40
	Right testicle	Before injection	5	13.88	4	0.00	3.40
		7th day	5	10.61	4	0.00	2.60
		14th day	5	5.88	4	0.00	2.20
		21st day	5	-	-	-	0
Group IV	Left testicle	Before injection	5	13.88	4	0.00	3.40
		7th day	5	5.09	4	0.007	2.60
		14th day	5	5.71	4	0.005	1.40
		21st day	5	5.71	4	0.005	1.40
	Right testicle	Before injection	5	19.00	4	0.00	3.80
		7th day	5	11.00	4	0.00	2.20
		14th day	5	5.71	4	0.005	1.40
		21st day	5	6.53	4	0.003	1.40

The information contained in Table 3 in the second group showed that:

1. Left testicular echogenicity prior to the injection is significantly higher than days 7 and 14.
2. The right testicular echogenicity prior to the injection is significantly higher than day 7.

In additional, Table 3 shows that echogenicity of the testicles significantly reduced in the third group. Also show that the left testicular and right testicular echogenicity reduced significantly during the experiment in the group IV($P < 0.05$).

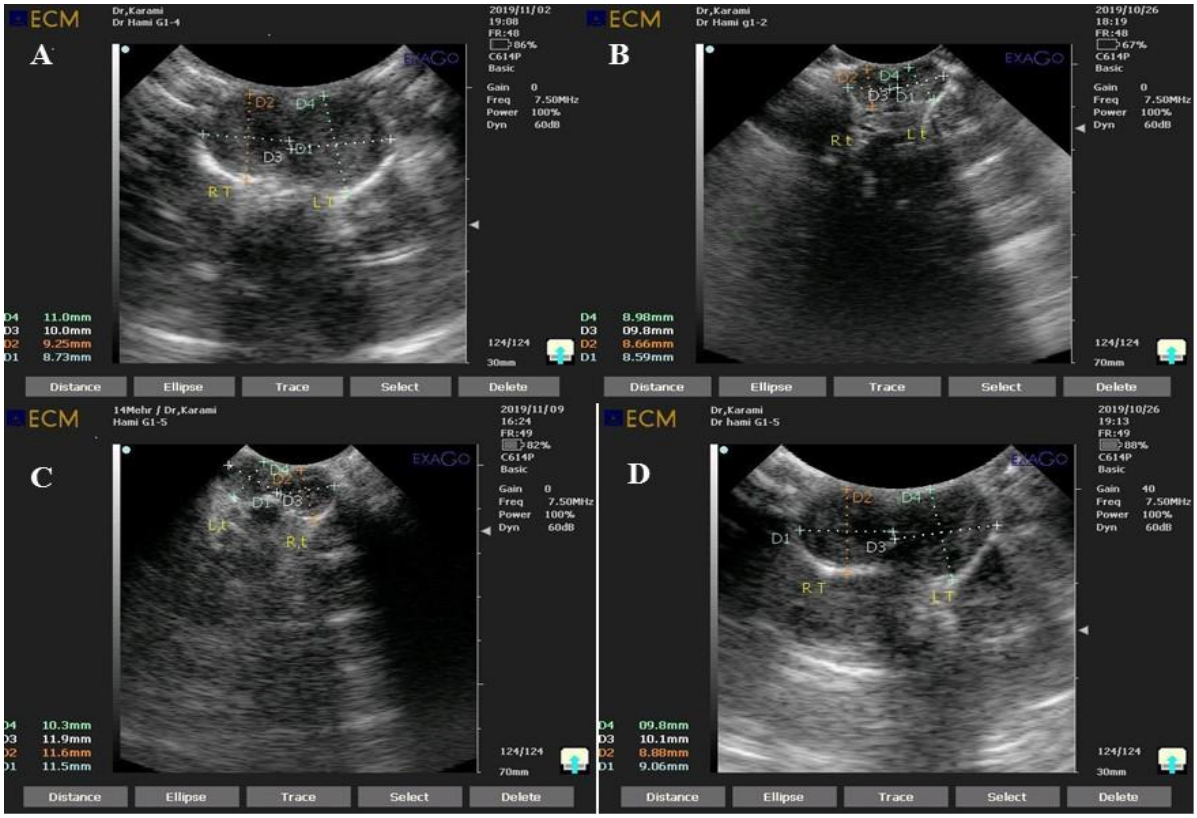

Fig.1: B-mode Ultrasonography of the left and right testis of rats. A: Measure the length and width of the left and right testicles prior to the injection of calcium chloride solution. B: Measure the length and width of the left and right testicles 7 days after injection of calcium chloride solution. C: B-mode Ultrasonography of the left and right testis of rats. A: Examination of the left and right testicles prior to the injection of calcium chloride solution. D: Evaluation of left and right testicular echogenicity 7 days after injection of calcium chloride solution.

b. Histopathology:

The results of the left testis tissue between the three groups mentioned in the tables and lines as follows:

Table 4. Mean and standard deviation of the left testicular damage caused by pathological examination of 15 mice

Damaged	Number	Group II		Group III		Group IV	
		Mean	Standard deviation	Mean	Standard deviation	Mean	**Standard deviation**
Intravascular thrombosis	5	1.20	0.73	1.60	0.89	1.60	**0.54**
Seminiferous Tubule necrosis	5	1.40	0.54	2.00	0.70	3.00	**1.00**
Bleeding	5	1.00	0.70	1.40	0.54	2.00	**0.70**
Interstitial Neutrophilic infiltration	5	1.60	0.54	1.20	0.83	3.20	**0.83**
Calcification	5	1.60	0.54	1.80	0.44	3.40	**0.89**

Table 5. Statistical differences between groups were observed damage (Intravascular thrombosis, Seminiferous Tubule necrosis, Bleeding, Interstitial Neutrophilic infiltration and Calcification)

Damaged	Sum of means	df	F	Sig
Intravascular thrombosis	7.73	14	0.44	**0.65**
Seminiferous Tubule necrosis	13.73	14	5.44	**0.02**
Bleeding	7.73	14	2.93	**0.09**
Interstitial Neutrophilic infiltration	18.00	14	9.88	**0.003**
Calcification	14.93	14	11.23	**0.002**

The information contained in Table 4 and 5 shows that:
1- Intravascular thrombosis factor (p = 0.65) and Bleeding factor (P = 0.09) between the three groups is not statistically significant.
2- In Seminiferous Tubule necrosis factor, there is a significant difference between the three groups (P = 0.02). Less mean is from Group IV (0.54) and highest mean is from group II (1.00).
3. Interstitial Neutrophilic infiltration factor between the three groups is a significant difference (P = 0.003). The lowest are from the group IV (0.54) and groups II and III are also the same mean (0.83).
4- There is a statistically significant difference in Calcification factor between the three groups (P = 0.002). So that the average in the group III (0.44) is lower than the group IV (0.54) and the group II (0.89).
It is noteworthy that the testis of the control group on 21st day was not seen any of the above injuries.

DISCUSSION

The results of the Ultrasonographic study in this study indicate that injection of calcium chloride solution reduces the size (length and width) and echogenicity of the testes over time so that on the 21st day almost the testicles are destroyed. Histopathological results also showed that calcium chloride injection caused multiple damage to testicular tissue. The most important damages are: Intravascular thrombosis, Seminiferous Tubule necrosis, Bleeding, Interstitial Neutrophilic infiltration, Calcification.
Various studies have been conducted to investigate the effects of this substance on chemical castration. For example, Jana et al. (2011) investigated the effects of 10% and 20% calcium chloride in saline solution with Lignocaine Hydrochloride in cats. Their results suggested that testicular tissue completely necrosis on day 60 after injection and is replaced by fibrous tissue [23].
Pereira et al. (2018) injected 20, 30 and 40% calcium chloride with a volume of 10 ml into the testes of 24 adult bulls. In histological evaluation, coagulation necrosis of seminiferous tubules and interstitial cells, infiltration of inflammatory cells, fibroplasia were widely seen in the tissue. They also stated that the concentration of the chemical is effective in the effects of testicular tissue, so that calcium chloride 40% has the greatest effect on testicular tissue. This finding is consistent with a study by Martins et al. (2011). They did not achieve complete testicular fibrosis using 30% calcium chloride solution in buffalo [24,25].
Similar studies of chemical castration with other substances include the study of Canpolat et al. (2016). They performed ultrasonography and histopathology of the effects of 20% sodium chloride on two groups of dogs (6 adult dogs and 6 immature dogs) The Ultrasonographic results of their study showed that chemical injection during 3 weeks significantly reduced testicular size, It was observed in immature dogs, but no significant changes were seen in adult dogs. They also reported an increase in echogenicity and echocardiography of testicular tissue and the presence of hypovascular points in the testis in both groups. Severe and extensive degenerative changes were seen with the presence of Leydig cells in the lumen [17].
But some researchers, such as Ahmed et al. (2016), do not consider chemical castration to be a viable alternative to testicular resection surgery. In their study of donkeys, they acknowledged that although injections of calcium chloride into the testicles lead to changes in testicular tissue (such as the presence of neutrophils and intratubular fibrosis); But because it does not reduce serum testosterone levels, it cannot be a statute for castrating animals [26].
Various studies have also shown that injection of a chemical in different animals may have similar results. For example, intra-testicular injection of glycerol in Sciurus saimiri causes sustained azoospermia [7]. But injecting this substance in the dog not lead to azoospermia and infertility [27].

CONCLUSION

Calcium chloride due to the numerous advantages suitable alternative to surgery. Among those benefits include cost-effectiveness, eliminating the need for anesthesia and sterile conditions and take care of recovery, less training and no effect on animal nutrition, interoperability with large populations and permanent (irreversible) sterilization. Calcium chloride can also be a more appropriate alternative to other chemicals, including benefits include a lower risk of skin necrosis in the development of less toxicity.
However, the time of effectiveness of this method is slow (approximately 2 to 3 weeks), which in later studies can be eliminated by changing the volume and concentration of calcium chloride. Examining the serum concentrations of sex hormones can also improve our understanding of this area.
In the end, it should be remembered that ultrasonography is also appropriate diagnostic tool that can be used to examine the process of testicular analysis.

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Conflict of interest
The authors declare that there isnoconflict of interest

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