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Chemical Sterilization by Intratesticular Injection of Eugenia Caryophyllata Essential Oil in Dog: A Histopathological Study

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Abstract

Objective- To study a method of chemical sterilization and its efficacy in adult male dogs.
Design- Experimental study.
Animals- Ten healthy adult mixed breed dogs
Procedures- Eugenia caryophyllata (EC) essential oil was injected into the dorsal cranial portion of each testicle of five dogs (treatment group). The same volume of normal saline was injected in the same site of testicles in the other five dogs (control group).
Results-There were no significant adverse effects and no change in the dog behavior during the study. Histopathological findings showed total necrosis of testicular tissue with fibrosis and hyalinization in seminiferous tubules and interstitial spaces. Infiltration of leucocytes was also observed. The serum concentration of testosterone was decreased significantly in treatment group. There was also no significant change in the serum concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), blood urea nitrogen (BUN), creatinine (CRE), total protein (TP) and cortisol level.
Conclusion and Clinical Relevance- Single intratesticular injection of an appropriate dose of EC can result in sterilization, which is preferable to surgical castration in dog.
Key words- Dog, Essential oil of Eugenia Caryophyllata, Chemosterilization, Testis, Testosterone.

Introduction

Eugenol (4-allyl-2-methoxyphenol) is the main component of oil of cloves *Eugenia caryophyllata* (syn. *Syzigium aromaticum*).[1] Essential oil extracted from the cloves contains almost 72–90% eugenol. Cloves are widely grown in Indonesia, Madagascar and also in other countries like India and Sri Lanka. Eugenol is a natural phenolic compound is present in reasonable amounts in several other spices like basil, cinnamon and bay leaves. Eugenol has been used as a flavoring agent in cosmetics and food products and also plays a role in dentistry as cavity filling cement. Eugenol is said to possess various biological properties like antiviral, antioxidant, anti-inflammatory, *etc*. At low concentrations, it usually acts as an antioxidant and anti-inflammatory agent, whereas at higher concentration, act as a pro-oxidant causing increased generation of tissue-damaging free radicals. It has beenreported to possess antigenotoxic activity[1] . Numerous studies have indicated that eugenol is cytotoxic to mouse fibroblast cell line L929[2], rat hepatocytes[3], pulp cells[2,4] and oral mucosal fibroblasts[5] in vitro.[6] Eugenol was also found to cause injury to rat oral mucosa membranes in vivo.[6,7] In most of Europe and the USA, wildlife (eg, raccoons, bats, and skunks) is the most important source of rabies whereas in Asia and Africa dogs are the primary vector of rabies and the biggest threat to humans; dogs have been the focus of the World Health Organization's rabies control program. Canine overpopulation and stray dogs represent a worldwide problem, compromising public health and animal welfare.[8] This problem has a negative influence on environmental hygiene and zoonosis.[9] Of all contraceptive methods for canine male population control, surgical sterilization is the most known and performed.[9,10] Some restriction with the method might occur from owners who consider this technique not compatible to animal welfare.[11] Research to develop chemical and hormonal alternatives that can be implemented by para-professional staff have a long and complicated history.[12,13] They may be of value in

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regions where cultural and religious beliefs prevent canine population control by culling, and where skilled veterinary surgeons are not readily available.[12]

For the last few years, researchers have been interested in developing a method for nonsurgical chemo-sterilization, as this process is suitable for mass-scale application and may be a better alternative to surgical castration.[14] Various agents have been used for nonsurgical chemical sterilization of male animals: the injection of steroid hormones including androgens[15], progestagens[16], antiandrogens[17], anabolic steroids[18] and androgens plus progestagens[19] in many species. However, these treatments do not consistently result in sterility. Intratesticular injections have been investigated as a method of inducing spermatogenic orchitis and male contraception for more than five decades.[20] Chemical orchidectomy with chemical agents has been suggested as a fast and low cost alternative that can be used in a wide range of canine population, especially in poor regions where the problem is more intense.[9] Chemo sterilization was experimented in males monkeys, hamsters, rabbits, rats and dogs by intratesticular injection of some agents such as ferric chloride, danazol, BCG, zinc tannate, glycerol, glucose, NaCl, dibromochloropropane[21], lactic acid[22], zinc arginine, sodium fluoride, formaline, calcium chloride[23], ethanol , and potassium permanganate plus glacial acetic.[12,24]

The purpose of the study was to determine the efficacy of intratesticular injection of essential oil of Eugenia caryophyllata (EC) for chemo sterilization in dogs.

Materials and methods

Preparation of essential oil of Eugenia caryophyllata Fresh spice (Clove) was bought from a main market in Kerman, Iran. They were milled to fine powder with a clean electric blender. The essential oil was extracted by hydrodistilation.[25,26]

Animals

Ten healthy adult mixed breed dogs aged 3 to 5 years (based on dental characteristics) with a body weight of 15 to 19 Kg were selected. A clinical examination and hemogram was performed on all animals. Their previous medical records of reproductive problems or traumas were not available. They were routinely de-wormed and vaccinated prior to arrival in the animal housing area. The animal house had facility of proper light and normal temperature (ranging from 19^{o} C to 25^{o} C). Dogs were housed in indoor runs and received food and water ad libitum.

Experimental protocol

Chemical concentration was made as follows: Group I: 5 dogs (control, saline solution); Group II: 5 dogs (EC).

All animals were examined for testicular size performed 15 days before chemical injection, on Day 0 and then every 7 days for 42 days. Body temperature, cardiac and respiratory rhythms and mucosal color were taken daily on each animal. Animal were checked for signs of pain, licking at the injection site and appetite.

The treatment group received a single bilateral intratesticular injection of essential oil of Eugenia caryophyllata, given in 1 mL solution containing 1% lignocaine hydro chloride (Pasteur Institute of Iran). The control group received a single bilateral intratesticular injection of 1 mL sterile normal saline which contained 1% lignocaine hydrochloride.

Each intratesticular injection was performed using a sterile 21-gauge needle directed from the caudoventral aspect of each testis approximately 1 cm from the epididymal tail and towards the dorsocranial aspect of that testis so that the solution was deposited over the entire route by linear infiltration while withdrawing the needle from the proximal end to the distal end.

Collection of blood and testes

Forty-two days following intratesticular injection, both testes were obtained from the dogs by castration[14], for histophological studies. Blood sample were taken before castration in fasted animals at 8:00 a.m.8:30 a.m.

The blood samples were centrifuged at 10,000 rpm for 15 min and the clear serum were collected and stored in a -20^{o}c freezer. The levels of testosterone, cortisol, alkaline phosphatase (ALP), lactate dehydrogenase (LDH), blood urea nitrogen (BUN), creatinine (CRE), total protein (TP) in serum were analyzed. The serum concentrations of testosterone and cortisol were measured using an ELISA reader (Merck, Japan) according to the standard protocol given by National Institute of Health and Family Welfare (NIHFW, New Delhi, India).[20] The ELISA kit for testosterone was supplied by IBL (Hamburg, Germany) and the cortisol kit was supplied by NIHFW. All biochemical assays were determined according to Pars Azmon Kits (Pars Azmon Co, Iran) by Autolab auto-analyzer (Rome-Italy). AST and ALT were assayed by the method of Reitman and Frankel.[27] ALP was assayed by the method of King and Armstrong.[28] LDH activity was evaluated according to the method by IFCC.[29] Total proteins of serum were measured according to the Biuret method of Alavi-Shoushtari et al.[30]

Histopathological Examination

The testicles were removed with open surgical technique 42 days after drug injection for histopathologic evaluation. The testes from each animal were fixed in 10% neutral buffered formalin and after the routine histopathological processing, tissue samples were embedded in paraffin. A section of 5 μm thick was cut from the middle portion of each testis, stained with

haematoxylin-eosin and examined under light microscopy at 200x magnification. The structures of the seminiferous tubules and interstitial spaces in the testis were examined.

Statistical Analysis

Measured values are reported as means ± standard errors (SEM), and statistical comparisons were performed using SPSS 17.0 statistics package (SPSS Inc, Chicago, IL, USA). The statistical analysis was done by using Student's 't' test. A p value of less than 0.05 was considered as significant.

Results

Plasma concentrations of testosterone and cortisol

Plasma concentrations of testosterone were decreased significantly ($P < 0.05$) in treatment group in comparison to control. There was no significant difference in cortisol level between the two group ($P > 0.05$; table.1).

Biochemical Evaluation

Serum biochemical data are presented in table 1. In general, EC essential oil treatment was well tolerated and there were no significant differences in measured serum biochemical parameters between the two groups ($P > 0.05$; Table1).

Histopathology

Testicular sections showed normal arrangement of germ cells in seminiferous tubules with distinct peritubular space and interstitial cells of Leydig in the control group.

Clove essential oil treatment induced severe degenerative changes in testicular tissue both in seminiferous tubules and interstitial cells of leydig. However, after EC essential oil treatment, different degrees of degenerative changes in testicular parenchyma were observed (Fig.1,2). Histopathological analysis of the testis of the animals treated with intratesticular injection of EC essential oil resulted in necrosis of the germinal epithelium and the presence of only fibrous and hyaline tissues. Complete derangement of the tubular architecture without any distinct boundary between the tubular and extratubular compartments was noted. There were no mature or immature germ cells in testicular sections. There was no sign of regeneration in germ cells and interstitial Leydig cells (Fig.3).

Microscopic examination of testicles from EC essential oil treated dogs showed severe diffuse tubular necrosis along with varying degree of inflammatory response (Fig.3). Inflammatory cells consisted of mainly mononuclear cells. Intertubular oedema, fibrosis and haemorrhage were also detected. Some of the necrotic cells showed desquamation, or even calcification. Intertubular vessels were severely congested (Fig.1).

Table 1. Effect of single bilateral intratesticular injection of Eugenia caryophyllata essential oil (EC) on serum concentration of ALT, AST, ALP, LDH, BUN, CRE, TP, Cortisol and testosterone in male dogs 42 days after intratesticular injection.

Parameter Group	ALT (U/L)	AST (U/L)	ALP (U/L)	LDH (U/L)	BUN (mg/dl)	CRE (mg/dl)	TP (g/dl)	cortisol (µg/ml)	Testosterone (ng/ml)
Control	22.4±2.4	15.2±2	37.8±5.6	47±4.64	27.6±2.5	1.22±0.1	6.96±0.5	4.4±0.8	10.2±1.28
EC	23.35±1.7	13.85±1.3	39.3±3.2	48.85±2.4	25.8±1.6	1.13±0.06	6.7±0.23	4±0.39	0.26±0.06[a]

Data are shown as mean±SE, n =10.
[a] p<.001, as compared with their respective control.

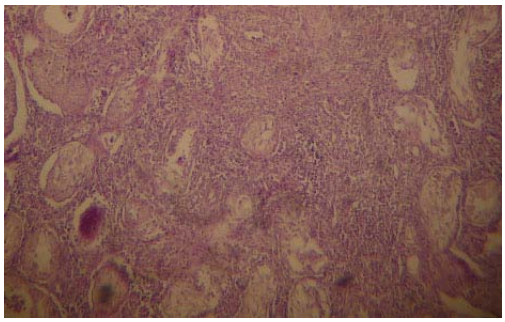

Figure 1. Photomicrograph of a testicular section 42 days after a single bilateral intratesticular injection of EC essential oil in a male dog. Showing disintegration of germ cell associations along with washing out of germ cells from the seminiferous tubules.

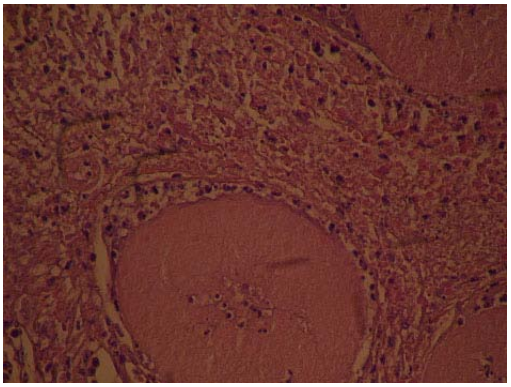

Figure 2. Photomicrograph of a testicular section 42 days after a single bilateral intratesticular injection of EC essential oil in a male dog. Showing complete derangement of the seminiferous tubular architecture along with the presence of fibrous tissue and hyaline tissue throughout the testicular parenchyma.

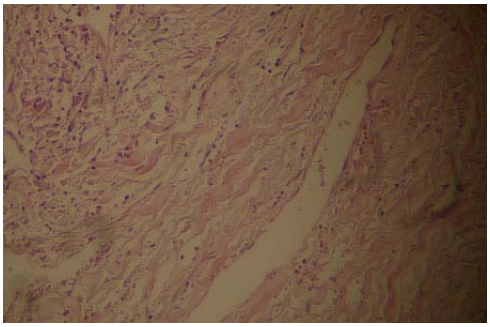

Figure 3. Photomicrograph of a testicular section 42 days after a single bilateral intratesticular injection of EC essential oil in a male dog. Showing complete necrosis of testicular parenchyma without any distinct boundary between the tubular and extratubular compartments along with the presence of fibrous tissue and hyaline tissue throughout the testicular parenchyma.

Discussion

Although intra-testicular injections have been investigated as a method of contraception in pet animals for more than five decades, this study is the first to show a potent EC essential oil intratesticular injection method in dogs and its mode of action. When permanent sterilization is desired in dogs, surgical castration can be expensive to be performed in a large scale. Moreover, this is the only study published so far reporting the sterilization of male dogs by single bilateral intratesticular injection of EC essential oil.

Injection of various agents into the epididymides[31,32] and testes[33] can help to limit male reproduction, although surgical castration is the most effective method. We found that a single intratesticular inejction of EC essential oil caused necrosis of testicular tissue in dogs.

A number of systemic routes of administration (intraperitoneal, subcutaneous, intravenous, and oral) are presently used for delivering agents to the testes to study their effects on spermatogenesis. Intratesticular injection is a technique that might offer some potential benefits, over other routes of administration, in the study of fertility control or regulation of spermatogenesis.[34] It may be used to study direct effects on the testis of agents of unknown toxicity, i.e., agents not systemically metabolized. A variety of chemical sterilants have been developed for injection into the testis and/or epididymides of dogs, which were either safe but not effective, or vice versa.[35] Numerous investigators have employed intratesticular injections to study a variety of agents.[34,36] Russell et al. in 1987 studied the technique of intratesticular injection and found that a sterile 26-gauge (or smaller) needle has no effects on the histology of the seminiferous tubules[37]. They suggested saline, ethylene glycol, dimethyl sulfoxide (DMSO) mixed 1:1 with saline, and propylene glycol to be suitable injection vehicles because they had no effect on testicular histology.[37] The first product obviously fulfilling both criteria was zinc gluconate; its utilization was described by Fahim et al in 1993[13] who injected it into the epididymides of dogs and tested intratesticular injection of zinc gluconate only in male puppies.[35]

EC has been used in traditional public medicine to relieve nasal obstruction and musculoskeletal pain which imply anti-inflammatory activity for the plant.[38] Analgesic, anesthetic, spasmolytic and antibacterial effects of EC were demonstrated by several scientific

studies.[38,39] Previous studies have reported that eugenol is a cytotoxic agent to mouse fibroblast cell line (L929), rat hepatocytes, pulp cells and oral mucosal fibroblasts in vitro. In addition, eugenol may injure rat oral mucous membranes in vivo.[6]

Besides providing valuable data for reproductive biology of this species, this study demonstrated that intratesticular injection of the EC essential oils altered the structure and function of the male reproductive tract, including: (1) lack of seminiferous germ cells; (2) morphological changes to Sertoli and Leydig cells; (3) atrophy of seminiferous tubules and (4) impairment of spermatogenesis. Based on these changes, we inferred that injection of EC essential oils caused infertility.

After EC essential oils treatment, complete degeneration of germ cells together with absence of a distinct boundary of seminiferous tubules with respect to the interstitial spaces along with appearance of fibrous tissue and hyaline tissue were observed. These changes may be due to the necrotizing properties of EC essential oils as reported by others.[20] In addition, germ cell degeneration by EC essential oils has been associated with low serum concentrations of testosterone, a prime regulator for the maintenance of structural morphology as well as the normal physiology of seminiferous tubules. [20]The low concentrations of serum testosterone in the groups treated with EC essential oils were further evidenced by the qualitative study of testicular sections in which significant fibrosis was seen with the treatment. These changes were due to low concentrations of testosterone.[23] These effects are consistent with previous studies using other chemical agents for chemo-sterilization.[40] Infiltration of leucocytes into the seminiferous tubules and interstitial spaces after treatment with the EC essential oils treatment may have been due to damage of the testicular tissue or to degeneration that may have released large amounts of chemotactic factors responsible for the ingression of leucocytes.[20,41]

The choronic stress indicators including concentrations of plasma cortisol, total plasma protein, blood urea nitrogen, creatinine and alkaline phosphatase were measured to ascertain whether the EC essential oils treatment was associated with any chronic stress response in the experimental animals.[42] Almost any type of stress will cause an increase in the secretion of cortisol in the dog.[14] Cortisol is established as an indicator of stress.[43] As there were no significant alterations in the plasma concentrations of cortisol, blood urea nitrogen, creatinine, alkaline phosphatase or total plasma protein concentrations in the animals treated with EC essential oils compared to the controls, this method of chemosterilization does not appear to be associated with any chronic stress response.[20,44,45] This single intra testicular injection of EC essential oils did not cause any general toxicity induction which has been reflected here by insignificant change in ALT and AST levels, as these are the main indicators of general toxicity.[46]

In conclusion, as the above evaluation was performed 42 days after intratesticular injection of EC essential oils, it indicates the possibility of irreversible chemo sterilization ability of the agent. The only proven methods for the sterilization of male dogs are orchiectomy and vasectomy. Both methods require anesthesia, adequate surgical facilities and equipment, and postoperative care. However, chemical castration is easy, effective, and permanent, and does not cause side effects. A single bilateral intra-testicular injection of EC essential oils is effective, economical, and easy to perform and does not require the removal of testis in dogs. The pathway to infertility is not clear from the present study, and it should be explored in the future. This agent may be used as a sterilizing agent for the control of the population of undesirable mammals, like stray dogs, after further investigation.

However, additional studies must be performed with a larger animal number and for a longer observation period in order to verify total security and absence of fertility recovery. Future studies should consider age, weight, breed, and previous medical history to minimize bias, which might have influenced our results.

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چکیده

عقیم سازی شیمیایی سگ با استفاده از تزریق داخل بیضه ای روغن اوژنیا کاریوفیلاتا (میخک): مطالعه هیستوپاتولوژیک

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هدف- مطالعه روش عقیم سازی شیمیایی و اثر بخشی آن در سگهای نر

طرح مطالعه- مطالعه تجربی.

حیوانات- ده قلاده سگ نر بالغ و سالم

روش کار- روغن گیاه اوژنیا کاریوفیلاتا (میخک) به داخل قسمت پشتی قدامی بیضه های پنج سگ گروه درمان تزریق گردید. حجم مشابهی از تزریق گروه درمان، سرم فیزیولوژی به داخل بیضه پنج سگ گروه کنترل تزریق شد. ارزیابی ۴۲ روز بعد از تزریقات صورت گرفت.

نتایج- مطالعات هیستوپاتولوژیک حاکی از نکروز کامل بافت بیضه همراه با فیبروز و تخریب لوله های اسپرم ساز و فضای بینابینیدر گروه درمان بود. نفوذ گلبولهای سفید نیز مشاهده گردید. کاهش شدید میزان تستوسترون سرم در گروه درمان نسبت به گروه کنترل مشهود بود. تغییرات ناشی از استرس عمومی مزمن در حیوانات مورد مطالعه دیده نشد به طوریکه تغییرات میزان آلانین آمینوترانسفراز، آسپارتات آمینوترانسفراز، آلکالین فسفاتاز، لاکتات دهیدروژناز، ازت اوره خون، کراتینین، پروتئین تام و کورتیزول سرم معنادار نبود.

نتیجه گیری و کاربرد بالینی- یک بار تزریق داخل بیضه ای عصاره گیاه میخک با دز فوق می تواند روشی قابل انجام در عقیم سازی سگهای نر بوده و جایگزین روش عقیم سازی جراحی شود.

کلید واژگان- سگ، عصاره گیاه میخک، عقیم سازی شیمیایی، بیضه، تستوسترون.