Egyptian Journal of Animal Health

P-ISSN: 2735-4938 On Line-ISSN: 2735-4946
Journal homepage: https://ejah.journals.ekb.eg/

Evaluation of spirulina efficiency on hormonal disruption induced by fipronil in Newzeland rabbits

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Received in 2/11/2022
Received in revised from 23/11/2022
Accepted in 7/12/2022

Keywords:
Spirulina
Fipronil
HPLC
Tissue-Rabbits

ABSTRACT

The current research assessed spirulina possible benefits effects in alleviating hormonal alterations and residue of liver and kidney fipronil and its metabolite in rabbits treated with dermal administration of 1/20 of LD50 (354 mg/kg fipronil) three times /week for four weeks. Sixty male New Zealand rabbits were distributed to four equal groups, control, spirulina (10 g/kg of diet daily), fipronil (1/20 of acute dermal LD50) and fipronil plus spirulina groups.

The data showed that fipronil caused hormonal changes and increased residue in the liver and kidney by extending the duration. Fipronil plus spirulina rabbit group showed significant increase in serum levels of testosterone and luteinizing hormone (LH), as well as triiodothyronine (T3) and thyroxine (T4) but significantly decreased serum levels of thyroid stimulating hormone (TSH) in contrast to the fipronil group. Spirulina showed non-significant effect on fipronil and fipronil sulfone residue of liver and kidney.

INTRODUCTION

Numerous ecosystems have become contaminated as a result of the growth industrial, technology, and the widespread of use chemicals as insecticides, herbicides and pesticides. Despite the fact that these pesticides often produce far less environmental damage than exposed animals'mortality, however, lesser pesticide contamination might damage normal physiological functioning and biochemical sys-

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DOI: 10.21608/EJAH.2023.282267

tems of tissues via lowering cellular reactive oxygen species, ROS, scavenging potential **(De Barros et al. 2017 Abdel-Daim et al.(2019)**

Fipronil (Frontline, FIP) is widely used insecticide in agricultural, veterinary and public health purposes against cockroaches, beetles, ticks, fleas, termites, ants and rootworms. Fipronil, a phenylpyrazole insecticide, inhibits gamma-aminobutyric acid-ergic receptors and generates ROS (**Khan et al. 2015 Gupta and Anadón 2018)**.

It is permitted for use as an ectoparasiticide on canines and felines. Despite the fact that its effective usage in rabbits, there are several reports of death within a day or malnutrition symptoms, lethargy and convulsions in young rabbits being intentionally administered fipronil **(Cooper and Penaliggon 1997 Cutler 1998)**. Fipronil is a strong disruptor of endocrine mediators and has negative consequences on development, behavior, and reproduction. Furthermore it has negative impact on animal spermatogenesis by preventing mitochondrial function with DNA destruction (**Udo et al. 2014 Badgujar et al. 2015 Khan et al. 2015 Badawy et al. 2018)**.

Genotoxic and reproductive hazards are the most reliable endpoint for evaluating how various substances may affect health **(Ghaffar et al. 2014)**. Fipronil metabolites are also released in cow milk due to lipophilic characteristics **(Le Faouder et al. 2007 Khan et al. 2015)**. According to studies **(Ohi et al. 2004 De Oliveira et al. 2012)**, fipronil could injury testicles, thyroid and liver tissues notably in mice and quail **(Badgujar et al. 2015 Mohammed et al. 2016)**.

Spirulina, cyanobacteria blue green algae, is among the oldest life forms on world. The FDA (United States) and the Brazilian Health Surveillance Agency both consider spirulina to be generally recognized as safe. Spirulina is widespread usage for its high protein, carbohydrates, fiber and antioxidant content (minerals, vitamins and phenolic acids) **(Salmean et al. 2015; Kata et al. 2018 Alghonaim et al. 2022)**. Because of its high protein content (up to 65%), polyunsaturated fatty acids, polysaccharides, carotenoid, glycolipids, vitamins (A, E, and B) and minerals (iron, potassium, iodine, magnesium, calcium, zinc, and manganese), spirulina has exceptional nutritional and health advantages **(Meineri et al. 2009 Torres-Duran et al. 2014 Moor et al. 2017)**.

Owing to biological diversity and high micronutrients content of spirulina, our research applied to examine spirulina ameliorative effect against some thyroid and reproduction hormones and estimation of fipronil and its metabolite (Fipronil sulfone) residue in liver and kidney tissues post topical application of fipronil three times per week for four weeks in adult male rabbits.

MATERIALS and METHODS

Animals:

Sixty adult, white, male New Zealand rabbits weighing between 3 ±0.25 Kg were saved in stainless steel cages and free access to water and ration (**NRC 1977**) with equally alternated between light and darkness illumination.

Supplies:

The Alga Biotechnology Unit, National Research Center, Dokki, Egypt, provided the spirulina powder, and the dosage for rabbits was 10 g/kg of diet by physically mixing 1% spirulina with the components of ration then formed pellets **(Peiretti and Meineri 2009)**.

The dose of 17.7 mg/kg equally 1/20 of acute dermal LD_{50} (354 mg/kg) in rabbits, fipronil 5% (BARS®, AVZ animal health, Russia) was topically applied on the base of the neck (**FAO, WHO, 1997**).

Experimental design:

Rabbits was divided into four equal groups; control, spirulina (10 g/kg of diet daily), fipronil (1/20 of acute dermal LD50 three times / week for four weeks) and fipronil plus spirulina groups.

Biochemical analysis

Serum testosterone and thyroid hormones (thyroxine; T4&triiodothyronine; T3) were estimated by simple and precise high-pressure liquid chromatography (HPLC) method as described by **Häkkinen et al. (2018)**. Using

3,3′,5-triiodo-L-thyronine (T3) (≥95%), L-thyroxine (T4) (≥98%), and testosterone (≥99%) and also formic acid and ethyl acetate that were purchased (Sigma-Aldrich, St. Louis, MO, USA) while methanol and HPLC water from MERCK (Madrid, Spain). All chemicals were HPLC grade. Preparation of calibration standards and quality control (QC) were described as **El-Sheshtawy et al. (2021).**

ELISA kit from Jérémy Decourtye, Repropharm Vet, Nouzilly, Franceand ELISA kits supplied by Monobind Inc. Lake Forest CA 92630, USA were used for serum luteinizing hormone (LH) and thyroid stimulating hormone (TSH) estimation, respectively manufacturer's instructions (**Ma et al. 2006**).

Tissue residues of fipronil plus its metabolite (fipronil sulfone) were determined using HPLC method (**Leghait et al. 2009**) by using Fipronil PESTANAL® (`95.0%) and Fipronil sulfone (PESTANAL®,98.0%), the analytical standards (Sigma Aldrich Company) were prepared in acetonitrile and stored at -20°C. The mobile phase (methanol–acetic acid 0.005N (67:33, v/v) was pushed at 0.8 ml/min flow rate. The separation was performed at 275 nm using C18 column (5 µm, 125 mm× 4.6mm). Homogenized tissue samples weighing 1 gm were mixed with 10 ml of 0.1% acidified acetonitrile, vortexed for 1 min at maximum speed, and then adds 4 g of MgSO4 (anhydrous) and 1 g of NaCl with vortexed for a minute then centrifuged the mixture at 4000 rpm for 10 min at 4°C. 1ml of the acetonitrile layer was transferred to 5ml tubes containing 25 mg of PSA sorbent and 150 mg of anhydrous MgSO4 and shaken for 5 minutes by hand and then centrifuged at 4000 rpm for 5 minutes. Samples were purified and filtered according to **Salim (2020**). For analysis by HPLC, 0.5 ml of the extract was set into amber vials.

Statistical analysis

The data were analyzed and expressed as the mean±SE for each group using one-way analysis of variance. Significant difference was at $P < 0.05$.

RESULTS

Intra-lab verification of analytical methods

For the assays for testosterone, thyroid hormones, fipronil, and fipronil sulfone, the analytical procedures were verified in accordance with **USP (2019),** and the findings were reported in table (1) and figures (1&2) demonstrating the method's verification results.

Table 1. Parameters of intra-lab verification for HPLC assays

Parameters	Testosterone	T3	T4	Fipronil	Fipronil sulfone
Range (ppb)	0.5-20 ng/ml			5-1000 ng/gm	
Retention time (min.)	7.9	5.699	6.213	11.474	12.635
LOD	0.04	0.01	0.03	0.008	0.036
LOQ	0.10	0.03	0.08	0.025	0.109
Recovery (%)	92- 96	94- 97	92- 94	95- 97	95-99

LOD = Level of detection
LOQ = Level of quantitation

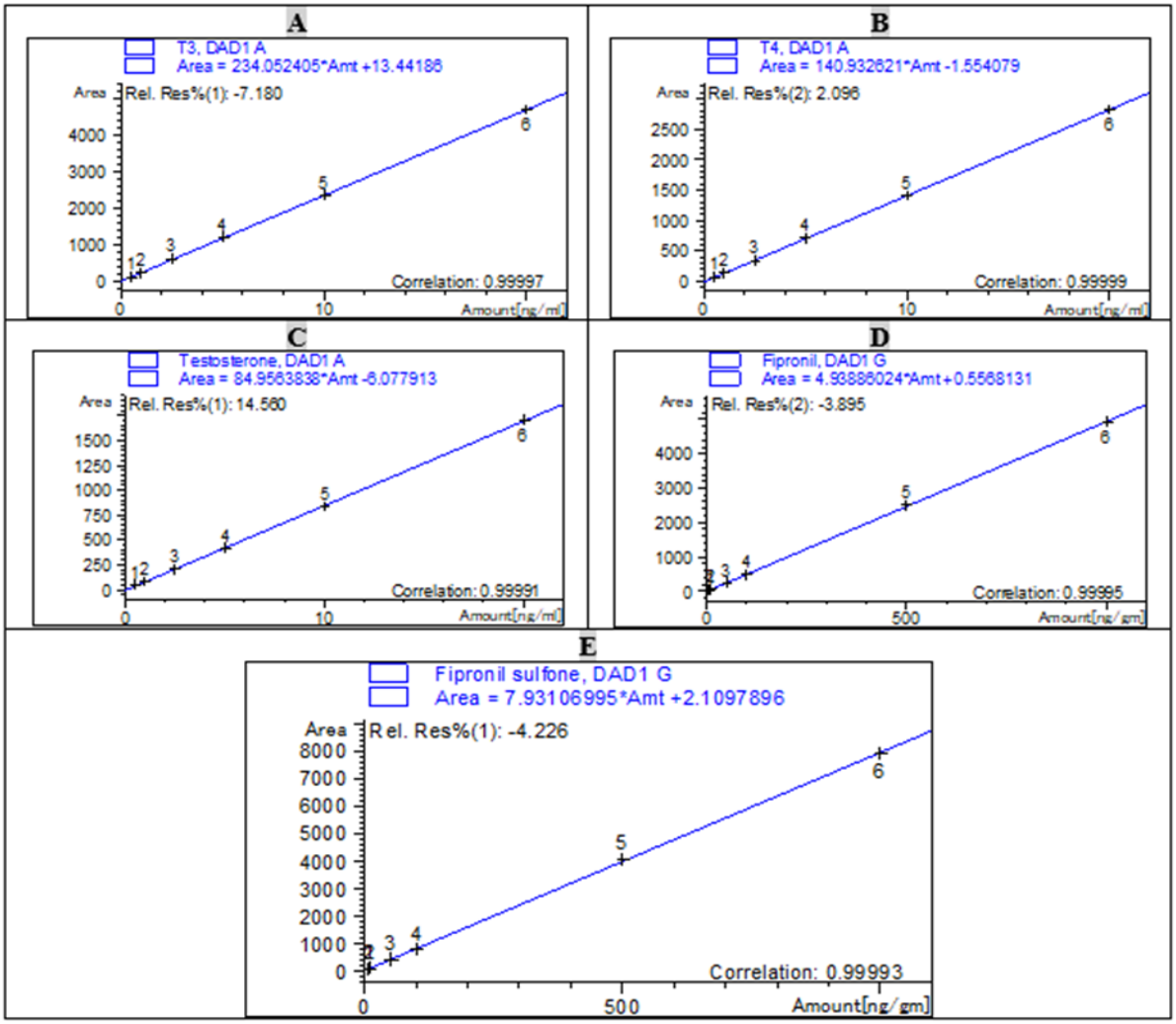

Figure (1): Standard curves of T3, T4 & testosterone hormones and fipronil & its metabolite; A, B, C, D&E; in progress.

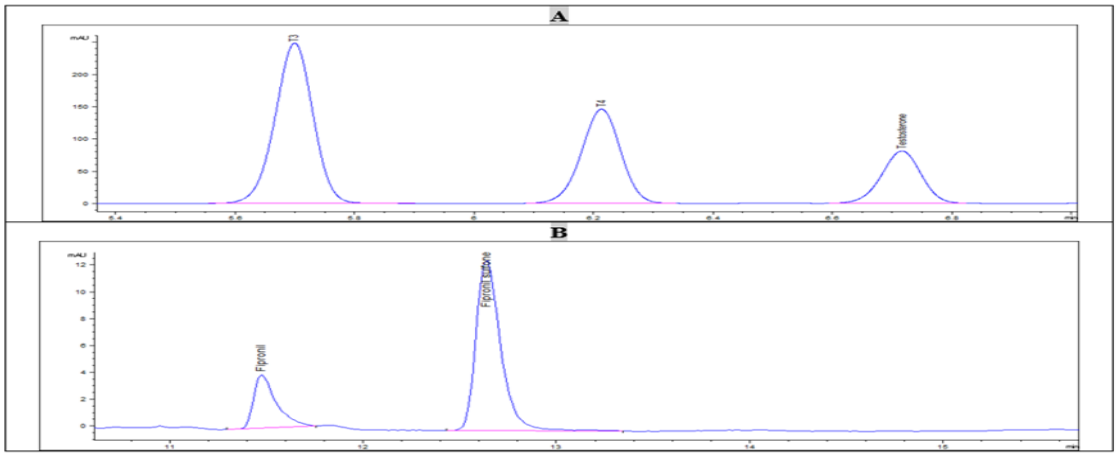

Figure (2): Chromatograms of T3, T4, &testosterone hormones [A], and of fipronil and its metabolite [B] at level of 5 ppb

Endocrinology results

Table (2): Mean ± SE of some serum hormones of different rabbit groups.

Groups	day	Control	Spirulina	Fipronil	Fipronil plus Spirulina
Testosterone (ng/ ml)	14	2.16± 0.008 [a]	2.21± 0.12 [a]	0.57± 0.046 [c]	0.93± 0.11 [b]
	21	2.16± 0.004 [a]	2.34± 0.14 [a]	0.44± 0.046 [c]	1.37± 0.16 [b]
	28	2.2± 0.09 [a]	2.48± 0.15 [a]	0.22± 0.036 [c]	1.38± 0.19 [b]
LH (mIU/ml)	14	21.36±3.15[a]	25.12±3.41[a]	7.65± 3.42[c]	14.83± 1.35 [b]
	21	20.48± 3.32 [a]	25.52±2.57[a]	6.34± 2.23[c]	14.56± 1.38[b]
	28	21.13±1.22[a]	25.79±2.47[a]	11.35± 4.13[c]	18.75± 2.14 [b]
Total T3 (ng/ml)	14	2.27± 0.21 [a]	2.33± 0.14 [a]	1.48± 0.09 [c]	1.88± 0.19 [b]
	21	2.27± 0.22 [a]	2.33± 0.15 [a]	1.49± 0.11[c]	1.83± 0.17 [b]
	28	2.27± 0.21 [a]	2.57± 0.14 [a]	0.14± 0.06[c]	1.71± 0.04 [b]
Total T4(ng/ml)	14	55.88± 0.44 [a]	59.27± 1.69 [a]	38.86± 2.03 [c]	52.58± 2.02 [b]
	21	56.23± 0.84 [a]	59.23± 1.52 [a]	34.76± 0.94 [c]	52.97± 1.96 [b]
	28	56.41± 0.66 [a]	64.5± 1.65 [a]	37.85± 1.02 [c]	57.68± 2.13 [b]
TSH (µIU/ml)	14	0.011±0.003[c]	0.009± .001[c]	0.021±0.001[a]	0.015± 0.001 [b]
	21	0.014± .003[c]	0.011±0.001[c]	0.026±0.001[a]	0.019± 0.001 [b]
	28	0.016± .002[c]	0.012± .001[c]	0.028± .001[a]	0.021± 0.001 [b]

The different alphabetical letters in each raw were significantly (P≤ 0.05)

Data analysis in table (2) showed no significant differences between the control group and the rabbits receiving spirulina during the study in terms of serum concentrations of testosterone, LH, T3, T4, and TSH. However, fipronil administration led to significantly decrease testosterone, LH, T3, and T4 levels as well as higher TSH level as recorded in fipronil group compared to control group (p ≤0.05). Fipronil plus spirulina group showed a significant increase in the level of serum testosterone, T3, LH and T4 (p ≤ 0.05) with marked decrease in serum TSH concentrations at 14, 21 and 28 days compared to the fipronil group (table2).

Fipronil and fipronil sulfone residue results

According to table (3), fipronil and fipronil sulfone were identified in liver and kidney of rabbits that administrated repeated cutaneous dosages of the fipronil. Liver fipronil and fipronil sulfone concentration reached 0.81 ± 0.04 to 17.7 ± 0.46 and 25.53 to 291.27 ng/g, respectively in fipronil rabbit group while in the fipronil plus spirulina rabbit group, it ranged from 0.88± 0.05 to 17.67 ± 0.75 and 25.9± 1.06 to 297.17± 19.9 ng/g, respectively.

Table (4) showed that kidney fipronil and fipronil sulfone residue varied from1.13 ± 0.13 to 22.01 ± 1.21 and 31.9± 1.15 and 321.4 ± 10.91 ng/g, respectively in fipronil rabbit group and from1.23± 0.12to 23.67± 1.55and 32.04± 1.89 to 338.63± 11.7ng/g, respectively in fipronil plus spirulina rabbit group. Unfortunately, spirulina showed non-significant difference in fipronil and fipronil sulfone residue in liver and kidney residues as reported in table 3&4.

DISCUSSION

Male rabbits' hypothalamic gonadotropin-releasing hormone is known to stimulate steroid genesis by increasing pituitary LH production and release. Pituitary LH attaches to its receptor on the surface of Leydig cells to boost testosterone biosynthesis. Pituitary dysfunction is caused by disruption of cell membrane-mediated signal pathways involved in the release of LH. As a result, the drop in LH levels must logically lead to testosterone drop **(Atessahin et al. 2006 McVey et al. 2008)**.

The obtained results showed reduced serum levels of LH and testosterone after topical administration of fipronil three times a week for four weeks. These results proved that fipronil alters anterior pituitary to generate FSH and LH as well as testosterone via causing oxidative stress in the Leydig cells. The lowered LH level may result from a disruption of hypothalamic-pituitary axis negative feedback control **(Kovacs et al. 1997)**. Fipronil has an inhibitory impact on testosterone synthesis, which stops spermatogenesis, as well as pituitary gonadotropins (FSH and LH). Fipronil is an androgen receptor antagonist, directly challenging testosterone and dihydrotestosterone for androgen receptor, which reduces testosterone synthesis **(Kavlock and Cummings 2005)**. So, fipronil has a negative impact on fertility and is particularly damaging to reproductive function of animals by reducing testosterone and developing pathological changes via the androgen-dependent epididymis **(Saleh et al. 2020)**.

Thyroid hormones are essential for maintaining energy balance as well as growth and development due to control triiodothyronine and thyroxin metabolism which affect the regulation of thermoregulation, heart rate, digestion and muscular contraction **(Oppenheimer 1999 Abdelatif and Saeed 2009)**. Immune system activities and reproductive system development are significantly influenced by these hormones. Hypothalamic pituitary thyroid axis regulates secretion and metabolism of endocrine active chemicals **(Silva 2003 Bassett and Williams 2016)**.

Fipronil increases T4 clearance by stimulating the hepatic enzymes required for T4 breakdown, which contributes for the lower level of T4. Reduced T4 negative feedback rises TSH secretion **(Hurley 1998 Tingle et al. 2003 Das et al. 2006 Leghait et al. 2009)**. The variation in serum T4 and T3 levels might be due to a slower rate of T4 to T3 conversion in peripheral tissues because of the impact of endocrine-disrupting substances, which primarily alter the activities of 6-phosphogluconate dehydrogenase, 5-deiodinase and malic enzyme**(Diamanti-Kandarakis et al. 2009)**. Additionally, according to **Nittoli et al. (2021)** exposure to the organophosphate chlorpyrifos interferes with thyroid hormones and reduces testicular T3-signaling affecting the function of the testicles in mice. The effectiveness of spirulina is based on its ability to protect several organs against the harmful effects of numerous medications and chemicals. The obtained result was demonstrating the enhanced pituitary and Leydig cell activity because of vitamins and minerals contents of spirulina that lower oxidative stress and counteract testis pathological changes caused by fipronil. In consistent to those rats given 300 mg/kg b.wt of spirulina after suffering arsenic poisoning demonstrated a drop in oxidative stress, testicular damage, and aberrant sperm. In addition, spirulina administration to cadmium-intoxicated rats at 150 mg/kg body weight for 10 days dramatically increased the testosterone level **(Bashandy et al. 2016 Farag et al. 2016 Eleiwa et al. 2018)**.

Table 3. Mean ± SE of liver fipronil and fipronil sulfone residues (ng/gm) in fipronil and fipronil plus spirulina rabbit groups

Day	Fipronil group		Fipronil plus Spirulina group	
	Fipronil	Fipronil sulfone	Fipronil	Fipronil sulfone
14	0.81± 0.04c	25.53± 0.74c	0.88± 0.05c	25.9± 1.06c
21	1.97± 0.24b	119.33± 3.81b	2.01± 0.26b	122.5± 4.18b
28	17.7± 0.46a	291.27± 17.36a	17.67± 0.75a	297.17± 19.9a

The different alphabetical letters in each column were significantly ($P \leq 0.05$)

Table 4. Mean ± SE of kidney fipronil and fipronil sulfone residues (ng/gm) in fipronil and fipronil plus spirulina rabbit groups

Day	Fipronil		Fipronil plus Spirulina	
	Fipronil	Fipronil sulfone	Fipronil	Fipronil sulfone
14	1.13± 0.13c	31.9± 1.15c	1.23± 0.12c	32.04± 1.89c
21	2.68± 0.13b	175.63± 9.74b	2.81± 0.17b	174.23± 11.3b
28	22.01± 1.21a	321.4± 10.91a	23.67± 1.55a	338.63± 11.7a

The different letters (abcd) in each column were significantly (P≤ 0.05)

The marker residue of fipronil, according to the European Food Safety Authority (EFSA), is identified by sum both fipronil and its sulfone metabolite. In our investigation, the total amount of fipronil and its metabolite was higher than the permitted level in all groups as the maximum residue limit (MRL) was set at 5 ng/ gm **(EFSA, 2018)**.

In instance, rabbits are more sensitive to fipronil. It is fatal in spray application. The onset of toxicity delays to be 3-9 days after treatment at lowest dosage. Post topical application in rabbits and neurological signs appeared up to three days because fipronil penetrates the skin and reaches the systemic circulation. In vitro, the depth of transdermal penetration of fipronil among species raised over time. Fipronil seems to permeate rabbit skin up to ten times deeper than rat skin therefore escalation the risk factor for rabbit poisoning **(Myers and Christopher 1993)**.

The wide range of liver and kidney residue might be due to repeated exposure to fipronil which metabolizes into fipronil sulfone by hepatic CYP enzymes in several mammalian species. Fipronil sulfone is created four fold greater in rat liver microsomes compared to human. Fipronil sulfone is a more potent inducer of hepatic enzymes and further cytotoxic to hepatocytes than fipronil. The sulfone is more tenacious in the organs than fipronil **(Mohamed et al. 2004 Das et al. 2006 Roques et al. 2012)**.

CONCLUSSION

Fipronil was recorded adverse effects on normal endocrine system function confirmed by thyroid, TSH, testosterone, LH, disruption in the rabbits. The administration of spirulina is fit for minimizing fipronil toxic side effects performed by hormonal alteration without effect on fipronil and fipronil sulfon accumulation in liver and kidney tissue of rabbits. Further investigation is needed to estimation of withdrawal period of fipronil residue in rabbit tissues

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