The Effect of Carbofuran on Thyroid Gland of Male Rats

Siti Nurma Hanim Hadie¹, Othman Mansor², Sharifah Emilia Tuan Shariff³

ABSTRACT

Objective: To investigate the effect of carbofuran which is a carbamate pesticide on thyroid gland by identifying the histopathological and histomorphometric changes, determining the thyroid activation index and quantifying the serum tri-iodothyronine (T3) and thyroxine (T4) level.

Methodology: This was a randomized control trial study. Eighteen Sprague Dawley rats between seven to ten weeks old were divided into control and intervention groups. The intervention group received 2.4 mg/kg Carbofuran orally for 28 days, while the control group received equivalent amount of vehicle. Sections of thyroid glands were sampled using the systemic uniform random sampling method and were stained with hematoxylin and eosin dyes. The serum tri-iodothyronine (T3) and thyroxine (T4) were measured by ELISA method.

Result: The study revealed abnormal thyroid gland in intervention group in terms of damaged follicles (p < 0.05), increased interstitial space (p < 0.05), abortive follicles (p < 0.001) and hyperplastic follicles (p < 0.05). Besides that, the colloid area and colloid circumference were found to be significantly higher (p < 0.05) while thyroid activation index and serum thyroxine (T4) were significantly lower in intervention group (p < 0.05).

Conclusion: Carbofuran induced thyroid toxicity by altering the follicular structure, reducing colloid resorption and reducing the serum thyroxine level.

KEY WORDS

carbofuran, histopathological changes, histomorphometric changes, thyroid activation index, thyroid hormones

INTRODUCTION

Carbofuran (2, 3-Dihydro-2, 2-dimethyl-7-benzofuranyl-N-methylcarbamate) is a broad spectrum systemic carbamate pesticide that kills insects, mites and nematodes on contact and after ingestion⁴. Carbofuran and other carbamate pesticides have widely replaced the more persistent and hazardous organophosphate pesticides used in agriculture due to its relatively short residual life in the environment and rapid degradation⁵. In Malaysia, it is widely used in paddy field, oil palm plantation and tobacco plantation for control of rodents and rhinoceros beetles³⁴. Owing to this, Carbofuran was reported to leach into the ground and surface water thus carrying the risk to various consumers and organism⁵.

Carbofuran acts by inhibiting the acetylcholinesterase enzyme but unlike the organophosphate pesticides, the activity is reversible⁶. It was reported that toxic signs of typical cholinesterase inhibition such as tremors, fasciculation, sedation, salivation and lacrimation occurred within minutes up to three hours after the administration⁵. The toxicity of acute and chronic Carbofuran exposure that had been documented in experimental animals includes reproductive and developmental toxicity, neurotoxicity, teratogenicity, mutagenicity and carcinogenicity. Carbofuran exert neurotoxic effect by impairing mitochondrial functions leading to oxidative stress. It produces signs such as exophthalmia, splayed hindlimb and staggered gait⁵. Gupta RC reported that Carbofuran could cross the placenta thus producing serious effect on the maternal-placental-fetal unit⁵. Besides that, it could induce disruption of estrous cycle in female hemiovariectomized albino mice, induces significant decrease in ovarian weight with concomitant decrease of compensatory ovarian hypertrophy and inhibits secretion of luteinizing hormone in rats which in turn affects the ovulation⁴⁰. It was also reported to cause reduction in epididymal sperm count and to decrease the libido in male rabbits and rats⁴⁰. Due to its effect on the reproductive system, Carbofuran has been listed as one of the endocrine disrupting chemicals. In general, endocrine disrupting chemicals affect mainly the sex steroid and thyroid hormones⁴⁰. However, a study done in male rats reported that Carbofuran only impairs the level of cortisol, estradiol and progesterone without altering the serum tri-iodothyronine (T3), total serum thyroxine (T4) and non-protein bound thyroxine (fT4) level⁴⁰. In contrast, different studies revealed that Carbofuran affects the thyroid system by causing significant increased in thyroxine concentration in ewes and induces adverse histophysiologial alterations in thyroid gland with impairment of the hypothalamo-neurohypophysial-seal-gonadal complex in teleost fish⁴⁰. In view of the inconsistent findings, the present study was carried out to investigate the effect of Carbofuran on thyroid gland by identifying the histopathological and histomorphometric changes, determining the thyroid activation index and quantifying the serum tri-iodothyronine (T3) and thyroxine (T4) levels after exposure to Carbofuran.

MATERIALS AND METHOD

Study Design

This study utilized the randomized control trial design.

Carbofuran and other chemicals

Carbofuran technical grade of 98 percent purity was purchased from Sigma Aldrich (M) Sdn. Bhd and dissolved in Sodium chloride 0.9% for oral administration. Other standard chemicals of highest purity were purchased from Sigma Aldrich, DAKO and Santa cruz biotechnology.
**Animals and ethical approval**

Eighteen male Sprague Dawley rats aged between seven to ten weeks old were purchased from the Laboratory Animal Research Unit of Universiti Sains Malaysia (LARU/SMU) and were randomly divided into the control and intervention groups. They were housed in six propylene cages with three rats per cage and had free access to standard pellet diet and water ad libitum. They were maintained with twelve hour light/dark cycle at room temperature. All rats were given individual identification numbers according to ear marking and cage number. To minimize stress responses, the rats were acclimatized for three days by daily handling and body weighing.

This study was carried out in compliance with the ethical approval from the Animal Ethics Committee, Health Campus, Universiti Sains Malaysia.

**Carbofuran administration**

The intervention group was given 2.4 mg/kg Carbofuran which was equivalent to 17% of the Carbofuran mean lethal dose (LD 50) in male rats, while the control group received equivalent amount of vehicle. The mode of administration was via oral gavage and it was carried out once daily in the morning for 28 days.

**Blood and serum preparation**

Twenty four hours after the last dose, the rats were euthanized by exsanguination technique via intracardiac puncture. The blood withdrawn from the right ventricle was kept in a plain tube and left for twenty four hours at two to eight degree centigrade prior to serum preparation. The serum was prepared by spinning the whole blood using a calibrated centrifuge machine and pipetted into plastic blood tubes. The serum was stored at minus 20 degree centigrade until hormonal analysis.

**Tissue collection and preparation**

The thyroid gland was excised together with the underlying trachea and fixed in 10% Formalin for 48 hours. Since the thyroid gland could not be separated from the trachea, the weight of the gland was not measured. The glands were embedded in paraffin wax into tissue blocks in perpendicular position to the cut surface and were serially sectioned at six µm thickness from base to apex. By adopting the systematic uniform random sampling methods, one sections was taken at every 30 sections (180 µm intervals) with random start between section number one and thirty. Finally, the sections were stained with hematoxylin and eosin dye.

**Histopathological analysis**

Six slides per sample were analyzed under a light microscope (Olympus, model CX31) for the presence of desquamation into follicular lumen, damaged follicles, increased interstitial space, presence of abortive follicles, hyperplastic follicles and hydropic degenerations.

By using control group as a guideline, a semiquantitive evaluation was performed by scoring the abnormality with ‘0’ for no change, ‘1’ for mild changes, ‘2’ for moderate changes and ‘3’ for marked changes. Each slide was analyzed in four different areas; therefore the total number of views analyzed for each sample were 24. For each view, the changes were marked either absent or present. No changes was defined as absence of the abnormality in all views. Mild changes was defined as presence of the abnormalities in one to eight out of 24 views. Moderate changes was defined as presence of the abnormalities in nine to sixteen out of 24 views. Marked changes was defined as presence of the abnormalities in 17 to 24 view.

### Table 1. Association between group variables with aggressive behaviour and loose stool

<table>
<thead>
<tr>
<th>Group</th>
<th>Aggressive behaviour</th>
<th>P-value*</th>
<th>Loose stool</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes (%)</td>
<td>No (%)</td>
<td></td>
<td>Yes (%)</td>
</tr>
<tr>
<td>Intervention</td>
<td>8 (88.9)</td>
<td>1 (11.1)</td>
<td>&lt; 0.001</td>
<td>5 (55.6)</td>
</tr>
<tr>
<td>Control</td>
<td>0 (0.0)</td>
<td>9 (100.0)</td>
<td></td>
<td>0 (0.0)</td>
</tr>
</tbody>
</table>

* Fisher exact test, significant at p < 0.05 as significant at 95%

### Histomorphometric analysis

Histomorphometric analysis was performed automatically by using a light microscope connected to a video camera and a computer equipped with image analysis software (Image ProPlus, Media Cybernetic). The analyses were done on four different test fields at 20X objective power for each section. For each view, five completed follicles were measured for histomorphometric assessment.

To avoid subjective selection of follicles, a lattice grid containing 25 small grids were placed on the computer screen. Each small grid was numbered from one to 25. Prior to follicle selection, five numbers representing a grid each were selected randomly by using Random Number Generator. The follicles that lay in the selected grids were used for morphometric measurement.

One hundred and eighty follicles for each rat were measured for follicle area, follicle circumference, colloidal area and colloidal circumference. For follicular epithelial cell height and area measurement, three cells from the follicles at twelve, four and seven o’clock positions were selected for the measurement.

### Thyroid activation index

Thyroid activation index was measured by calculating the ratio of follicular epithelial cells volume to colloidal volume. The volume density for follicular epithelial cells and colloidal by using the equation proposed by Hayat MA cited in Kmiec Z (18):

\[
V = BP^{1/2}.
\]  

In equation [1], V denotes volume density for epithelial cells or colloidal, B is the coefficient which is 1.85 for follicular epithelial cells and 1.382 for colloidal, and P is the section area for epithelial cells or colloidal.

### Hormonal analysis

Quantitative analysis of tri-iodothyronine (T3) and thyroxine (T4) were done by using rodent tri-iodothyronine and thyroxine enzyme immunosorbent assay kits (Endocrine technology, Newark, United States).

### RESULT

Throughout the experiment, three rats from the intervention group developed signs of cholinesterase inhibition but no toxicity-related deaths occurred. There was significantly higher proportion of rats developing aggressive behaviour and passing out loose stool in the intervention group compared to the control group (Table 1). For the mean body weight changes, no statistically significant difference was seen between the intervention and the control groups.

### Histopathological Changes

The histopathological changes identified were desquamation into follicular lumen, damaged follicles, increased interstitial space, presence of abortive follicles, hyperplastic follicles and presence of hydropic degeneration. The desquamation into follicular lumen was characterized by the presence of free cells inside the lumen (Figure 1(a)). Figure 1(b) shows damaged follicles which are characterized by joined follicles and disappearance of cellular boundaries. The increased interstitial space was found to be due to intestinal oedema and infiltration of fat cells (Figure 1(c) and 1(d)). Presence of abortive follicle was assessed by looking at the microfollicles with minimal colloid whereas the hyperplasia was determined by follicular cell crowding (Figure 1(e)). The features of hydropic degeneration are swollen...
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Figure 1. Histopathological changes observed in intervention group
(a) Desquamation into follicular lumen
(b) Damaged follicles (black arrows) and ectetic blood vessels (white arrows)
(c) Increased interstitial space by fat cells infiltration
(d) Increased interstitial space due to interstitial tissue edema
(e) Abortive follicles (black arrows) and hyperpalstic follicles (white arrows)
(f) Hydropic degeneration

Table 2. The comparison of severity score of histopathological changes between intervention and control group

<table>
<thead>
<tr>
<th>Histopathological changes</th>
<th>Severity score, Median (IQR)</th>
<th>Z Statistics</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intervetion</td>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desquamation into follicular lumen</td>
<td>1.00 (2.00)</td>
<td>1.00 (0.00)</td>
<td>-0.793</td>
</tr>
<tr>
<td>Damaged follicles</td>
<td>1.00 (1.00)</td>
<td>0.00 (1.00)</td>
<td>-2.698</td>
</tr>
<tr>
<td>Increased interstitial space</td>
<td>1.00 (2.00)</td>
<td>1.00 (1.00)</td>
<td>-1.966</td>
</tr>
<tr>
<td>Abortive follicles</td>
<td>2.00 (1.00)</td>
<td>2.00 (1.00)</td>
<td>-3.571</td>
</tr>
<tr>
<td>Hyperplastic follicle</td>
<td>2.00 (1.00)</td>
<td>1.00 (1.00)</td>
<td>-3.130</td>
</tr>
<tr>
<td>Hydropic degenerations</td>
<td>2.00 (1.00)</td>
<td>2.00 (2.00)</td>
<td>-1.148</td>
</tr>
</tbody>
</table>

*Mann-Whitney Test. P-value of < 0.05 as significant at 95% CI.

Table 3. Mean ± SD of follicle area, follicle circumference, colloid area and colloid circumference

<table>
<thead>
<tr>
<th>Histomorphometric features</th>
<th>Group</th>
<th>Area (μm²), Mean (SD)</th>
<th>t Statistics (df)</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intervention</td>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follicle area</td>
<td>352064.83 (82286.13)</td>
<td>289639.22 (59319.41)</td>
<td>1.846 (16)</td>
<td>0.083</td>
</tr>
<tr>
<td>Follicle circumference</td>
<td>2131.34 (231.74)</td>
<td>1958.18 (187.70)</td>
<td>1.742 (16)</td>
<td>0.101</td>
</tr>
<tr>
<td>Colloid area</td>
<td>199609.49 (61153.50)</td>
<td>135956.51 (43604.34)</td>
<td>2.542 (16)</td>
<td>0.022</td>
</tr>
<tr>
<td>Colloid circumference</td>
<td>1570.54 (227.17)</td>
<td>1336.13 (209.37)</td>
<td>2.276 (16)</td>
<td>0.037</td>
</tr>
</tbody>
</table>

*Independent-t test. P-value of < 0.05 as significant at 95% CI.
received 12 mg/kg body weight Carbofuran per day for a year. The rea-
posed that emesis and loose stool were seen in female beagle dogs which passed out loose stool and exposure to Carbofuran. Previous study reported that neostigmine, an acetylcholinesterase inhibitor.
by Beleslin DB and Samardžić R when laboratory animals were observed that the rats treated with Carbofuran developed irritable bowel syndrome in mice.

**DISCUSSION**

The present study reported that association between aggressive behaviour and exposure to Carbofuran was highly significant. It was observed that the rats treated with Carbofuran developed irritable type aggressive behaviour in which they became aggressive in the presence of hostile organism or objects. Similar finding was reported by Beleslin DB and Samardžić R when laboratory animals were exposed to neostigmine, an acetylcholinesterase inhibitor.
The present study also showed significant association between passing out of loose stool and exposure to Carbofuran. Previous study reported that emesis and loose stool were seen in female beagle dogs which received 12 mg/kg body weight Carbofuran per day for a year. The reason for passing out loose stool was probably due to the alteration in the motility of the bowel, which is primarily controlled by the myenteric plexus. Since the main neurotransmitter released by the myenteric neuron is Acetylcholine which exert parasympathetic effect to the bowel, the anti-cholinesterase effect of Carbofuran might have induced accumulation of Acetylcholine at both preganglionic and postganglionic parasympathetic neurons of the intestine. This accelerates the process of intestinal transit time which finally results in loose stool.

For histopathological changes, damaged follicles which indicate degenerative changes of thyroid gland were found to be statistically significant in the intervention group. There was also significant increase of interstitial space by fatty infiltration and edema in the thyroid gland of the intervention group and similar finding was reported by Selmanoglu G and Koçkaya EA and Barlas N et al in the thyroid of partulin and carbendezim-treated rats. This might be attributable to the hypothyroidism which leads to the accumulation of hyaluronic in the rats and adipocytes expansion in mice.

Besides that, it was also postulated that increased incidence of goitrogenic agents such as pesticides induced thyroid hyperplasia with abortive follicles in rats. The present study, stress induced follicular damage could not be ruled out.

There was also significant increase of interstitial space by fatty infiltration and edema in the thyroid gland of the intervention group and similar finding was reported by Selmanoglu G and Koçkaya EA and Barlas N et al in the thyroid of partulin and carbendezim-treated rats. In the present study, colloid area and circumference were significantly lower in the intervention group compared to control group (Table 3). Other variables were not significant.

**Histomorphometric Changes**

Table 3, 4 and 5 show the mean and standard deviation for histomorphometric variables. Independent-t test showed that the mean colloid area and cirumference were significantly higher in intervention group compared to control group (Table 3). Other variables were not significant.

**Thyroid Activation Index**

Table 6 shows the mean and standard deviation for thyroid activation index. Independent-t test revealed that the index was significantly lower in the intervention compared to the control group.

**Serum Tri-iodothyronine and Thyroxine**

The Mann-Whitney test was applied to determine the median of serum tri-iodothyronine (T3) and thyroxine (T4) in both groups. The median was significantly lower in the intervention group compared to the control group. This is in accordance with the findings of Capen et al cited in Khan MA et al who reported that significantly increased colloid area and low follicular cell height were associated with resting or inactive follicles. This finding corresponds with other features of the present study which suggest the idea of inactive follicles.

In the present study, colloid area and circumference were significantly larger in the thyroid gland of intervention group compared to control group. This is in accordance with the findings of Capen et al cited in Khan MA et al who reported that significantly increased colloid area and low follicular cell height were associated with resting or inactive follicles. This finding corresponds with other features of the present study which suggest the idea of inactive follicles.

Besides that the significantly lower thyroid activation index in the intervention group indicates that the thyroid glands were less active after exposure to Carbofuran. Previous studies proved that the index was found to be low in inactive thyroid gland of old rats and rats that were exposed to extremely low frequency electromagnetic field. For hormonal analyses, the serum thyroxine (T4) was significantly lower in the intervention group compared to the control group. Several animal-based studies reported that exposure to industrial chemicals and pesticides decreased the level of circulating thyroid hormone especially thyroxine (T4) and to lesser extend, tri-iodothyronine (T3). However, Barlas N et al reported that exposure to

**Table 4. Median and interquartile range of the follicular cell area**

<table>
<thead>
<tr>
<th>Features</th>
<th>Group</th>
<th>Z Statistics</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intervention</td>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>Follicular cell area</td>
<td>10189.08 (4478.22)</td>
<td>9296.57 (2091.81)</td>
<td>-0.132 (0.895)</td>
</tr>
<tr>
<td>cell area</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Mann-Whitney Test. P-value of < 0.05 as significant at 95% CI.

**Table 5. Mean ± SD of Follicular cell height**

<table>
<thead>
<tr>
<th>Features</th>
<th>Group</th>
<th>t Statistics</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intervention</td>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>Follicular cell height</td>
<td>81.21 (14.28)</td>
<td>85.28 (8.23)</td>
<td>-0.741 (0.469)</td>
</tr>
<tr>
<td>cell height</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Independent-t test. P-value of < 0.05 as significant at 95% CI.

**Table 6. Mean ± SD of Thyroid activation index**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group</th>
<th>t Statistics</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intervention</td>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>Thyroid activation index Mean</td>
<td>0.016 (0.009)</td>
<td>0.028 (0.014)</td>
<td>-2.196 (0.043)</td>
</tr>
</tbody>
</table>

* Mann-Whitney Test. P-value of < 0.05 as significant at 95% CI.

**Table 7. Median and Interquartile range of serum tri-iodothyronine and thyroxine**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group</th>
<th>Z Statistics</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intervention</td>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>Tri-iodothyronine</td>
<td>0.50 (0.50)</td>
<td>0.50 (0.30)</td>
<td>-1.060 (0.289)</td>
</tr>
<tr>
<td>Thyroxine</td>
<td>90.00 (28.80)</td>
<td>160.00 (142.50)</td>
<td>-2.700 (0.007)</td>
</tr>
</tbody>
</table>

* Mann-Whitney Test. P-value of < 0.05 as significant at 95% CI.

*Skewed to the right

parasympathetic neurons of the intestine. This accelerates the process of intestinal transit time which finally results in loose stool.

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CARBOFURAN AND THYROID GLAND

CONCLUSION

In conclusion, the present study showed that Carbofuran exert toxic effect to thyroid gland of male rats. Exposure to oral Carbofuran for 28 days caused alteration to follicular structures that lead to inactive follicles, reduced colloid resorption and reduction in serum thyroxine (T4) level. In order to improve the understanding on the effect of Carbofuran to thyroid gland, efforts should be made to investigate the possible mechanism for the changes of thyroid gland morphology. Besides that, future study should concentrate on measuring the Thyroid stimulating hormone (TSH) rather than measuring tri-iodothyronine (T3) and thyroxine (T4) since TSH level will provide better understanding of the thyroid hormone status.

ACKNOWLEDGEMENT

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