Thiamethoxam

Thiamethoxam - Overview of Testis Effects in Two Rat Multigeneration Reproduction Studies

Supporting Document for Submission

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1.0 OVERVIEW OF MULTIGENERATION REPRODUCTION STUDIES

The purpose of this document is to provide clarification on the results of the two multi-generation studies conducted on Thiamethoxam ([redacted] 1998, 2000; [redacted] 2004a). There were a number of technical deficiencies in the first study conducted at Novartis Crop Protection AG ([redacted] 1998, 2000), which triggered a second study at the Central Toxicology Laboratory (CTL) in 2003, incorporating a more comprehensive evaluation of endocrine parameters and testicular histopathology ([redacted] 2004a). Two control studies were conducted in parallel with this study, to provide information on the background variability of Tif:RAIf (SPF) rats, which were not a frequently used strain at the conducting laboratory ([redacted] 2004b,c).

1.1 1st Two-Generation Study: [redacted] (1998; 2000)

Groups of 30 male and 30 female rats were continuously fed diet containing (control), 30, 50, 1000 or 2500 ppm Thiamethoxam, in order to evaluate the effects on reproductive function. Compliant to GLP and the previous version of OECD TG 416 (1983), the Two-Generation Study in Tif:RAIf (SPF) rats ([redacted] 1998; 2000) was considered reliable without restriction, i.e. Klimisch 1 (Klimisch *et al.* 2007), and sufficient to assess the potential for Thiamethoxam to functionally impact reproductive function, mating, conception, gestation, parturition, lactation, weaning and pup development. However, there were a number of methodological flaws in the additional sperm parameters investigated as part of the study ([redacted] 1998), which triggered a subsequent focussed study to analyse sperm parameters ([redacted] 2000).

1.1.1 Sperm parameters

Sperm motility in F0 & F1 males of treatment groups was reduced significantly in all groups when compared to controls, in a non-dose related manner, with no apparent difference between the generations (Table 1).

Table 1: Sperm motility (%) ([redacted] 1998)

Generation	Dietary concentration of CGA293343 technical (ppm)				
	0	**10**	**30**	**1000**	**2500**
F0	73	55*	51**	56*	55*
F1	65	53*	55	60	53*

* Statistically significant difference from control group mean, $p<0.05$

The quality of sperm preparation medium has been shown to affect the motility, viability, and DNA integrity of spermatozoa (Anbari *et al.* 2016). Conducted prior to harmonised OECD guidance on sperm parameters, there were a number of methodological flaws in the [redacted] study (1998; 2000), reflected in the % motility of controls (F1 = 65%), which

failed to meet the minimum threshold considered acceptable (70% motility) (Seed *et al.* 1996; Parker 2012).

[redacted] (1998; 2000) collected the cauda epididymal sperm with a glass capillary tube (aspiration method), suspending in modified HAM's F10 medium. The aspiration method has been shown to significantly reduce percentages of motile and progressive sperm (Dostal *et al.* 1996), which led Working Groups to recommend the diffusion method for collecting sperm from the cauda epididymis (Seed *et al.* 1996; Chapin *et al.* 1992).

Critically dependent on temperature, sperm motility should be measured at 34 - 37°C, prior to the morphology or count. It is not clear whether [redacted] (1998) maintained the sperm samples at an appropriate temperature, nor how the medium was supplemented. Whilst buffered saline solutions at physiological pH are sufficient for sperm survival over short time periods (0 – 1 hour), there are differences in the abilities of media to support sperm survival over longer periods of time. Bovine Serum Albumin (BSA) levels of 0.5% to 1% are also necessary to support sperm motility (Seed *et al.* 1996). In the absence of an appropriate methodology and randomisation, in addition to the high degree of variability, as reflected by the large standard deviations, the toxicological relevance of the results on sperm motility are considered equivocal. This equivalence is supported by the absence of effects on the number of spermatids, morphology, fertility and fecundity across all groups in this study ([redacted] 1998, 2000).

Due to the inadequacy of the sperm analysis, an additional study was conducted to study the effects of Thiamethoxam on sperm cell parameters in 30 virgin male Tif:RAIf (SPF) rats, administered 0, 10, 30, 1000 or 2500 ppm in the diet for 4 weeks ([redacted] 2000). After 10 weeks, the animals were necropsied and the testes, epididymides, prostate, and seminal vesicles (with coagulating glands) were weighed, and epididymal sperm cells and/or testis spermatids were evaluated for number, motility, and morphology.

Contrary to the first sperm analysis ([redacted] 1998), a number of procedures were implemented to reduce and standardise the time for sperm collection, refine the technique for opening the cauda epididymis, and to ensure randomisation in order to minimise inter-day bias ([redacted] 2000). There were no treatment-related effects on the proportion of motile sperm cells within the cauda epididymis fluid, the proportion of morphologically abnormal sperm, the number of spermatids per gram of testis or the number of sperm cells per mg cauda epididymis fluid (Table 2). Whilst this second analysis ([redacted] 2000) did not assess the F_1 generation, no differences had been seen between the generations in the original study, and the inadequacies of the initial sperm assessment ([redacted] 1998) were clearly highlighted. This later analysis is considered a much more robust assessment of sperm parameters, and shows no effect of treatment.

Table 2: Sperm parameters (follow-up sperm analysis (reference))

	Dietary concentration of CGA293343 technical (ppm)				
Dose Level	**0**	**10**	**30**	**1000**	**2500**
Total spermatids ($x10^6$ /g testis)	59.6	58.2	55.0	57.5	55.8
Percent abnormal sperm	12.2	11.7	11.6	12.2	11.0
Percent motile sperm cells	73	76	73	74	74

No statistically significant differences from control group means

1.1.2 Testicular pathology

Subtle microscopic changes in the testes were seen in the F1 generation. Whilst the study report dismissed the minimal, focal, tubular changes in F1 males as not being treatment related, a re-evaluation was conducted at the request of Japanese regulatory authorities. This Pathology Working Group (PWG) re-evaluation, assessed against several control groups from the same laboratory, concluded that the increased incidence of tubular atrophy in F1 animals at 1000 and 2500 ppm was associated with thiamethoxam treatment, whilst findings at 30 ppm were considered equivocal.

Table 3: Incidence of testicular tubular atrophy in F0 and F1 males (PWG re-evaluation)

Finding	Dietary concentration of CGA293343 technical (ppm)									
	0	**10**	**30**	**1000**	**2500**	**0**	**10**	**30**	**1000**	**2500**
	F0 Males					**F1 Males**				
Minimal focal tubular change	15/30	12/30	12/30	15/30	13/30	12/30	13/30	17/30	19/30	18/30
grade 1	8/30	12/30	7/30	12/30	8/30	8/30	3/30	8/30	8/30	7/30
grade 2	7/30	0/30	5/30	3/30	5/30	4/30	10/30	9/30	11/30	11/30
%	50.0	40.0	40.0	50.0	43.3	40.0	43.3	56.7	63.3	60.0
Average grade	1.5	1.0	1.4	1.2	1.4	1.3	1.2	1.5	1.6	1.6
Diffuse tubular atrophy	1/30	1/30	2/30	3/30	2/30	0/30	1/30	1/30	7/30**	3/30
grade 2	0/30	0/30	1/30	0/30	0/30	0/30	0/30	1/30	2/30	0/30
grade 3	0/30	0/30	0/30	1/30	1/30	0/30	1/30	0/30	1/30	1/30
grade 4	1/30	1/30	1/30	2/30	1/30	0/30	0/30	0/30	2/30	2/30
grade 5	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	2/30	0/30
%	3.3	3.3	6.7	10.0	6.7	0	3.3	3.3	23.3	10.0
average grade	4.0	4.0	3.0	3.7	3.5	-	3.0	2.0	3.6	3.7

** Statistically significant difference from control group mean, $p<0.01$

Table 4: Consolidated incidence and severity of testicular tubular atrophy diagnosed by the PWG in F1 generation male rats

Dose	0 ppm	10 ppm	30 ppm	1000 ppm	2500 ppm
No. Exam.	30	30	30	30	30
Grade 1	6	7	9	8	10
Grade 2	0	3	4	8	5
Grade 3	0	0	0	1	1
Grade 4	0	0	0	2	1
Grade 5	0	0	0	2	1
Overall Incidence	6	10	13*	21**	18*
%Incidence	20%	33%	43%	70%	60%
Ave. Grade	1.0	1.3	1.3	2.1	1.8

*P 0.05; **P 0.01 (one sided Fisher Exact Test)

Table 5: Incidence and severity of testicular tubular atrophy diagnosed by the PWG in untreated F1 animals of five two-generation studies

Study number	951041	951031	943045	923152	923179
No. Exam.	30	30	30	30	30
Grade 1	5	9	1	2	3
Grade 2	1	2	1	2	2
Grade 3	0	0	0	0	0
Grade 4	1	0	0	0	0
Grade 5	2	0	0	0	3
Overall Incidence	9	11	3	4	8
%Incidence	30%	36.7%	10%	13.3%	26.7%
Ave. Grade	2.3	1.2	2.7	1.5	2.8

For studies of 13 weeks duration or less, the testes should be fixed with Modified Davidson's (or Bouin's) fixative for 24 – 48 hours, to avoid artefacts and differential tubular shrinkage (Parker 2012; Lanning *et al*. 2002; Creasy 2003). Subsequently, Periodic Acid Schiff (PAS) staining is recommended for accurate identification of the tubular stages of spermatogenesis (Lanning *et al.* 2002; OECD GD 16). Contrary to current guidance, [redacted] (1998; 2000) fixed the testes in 4% formalin and stained with haematoxylin and eosin (H & E). Whilst the histopathological assessment is considered reliable, it should be noted that the histology methodology was sub-optimal, which may have complicated the interpretation of testicular lesions.

1.2 2nd Two-Generation Study: [redacted] (2004)

Equivalent to OECD TG 416 (2001) and compliant to GLP and the US EPA OPPTS 870.3800 (1998), the Two-Generation Study in Tif:RAIf (SPF) rats ([redacted] 2004a) was considered reliable without restriction, i.e. Klimisch 1 (Klimisch *et al*. 2007), and provided a robust assessment of testis histopathology and sperm parameters. Groups of 26 males and 26 female (F0) rats were fed diet containing 0 (control), 20, 50, 1000 or 2500 ppm thiamethoxam. Consistent with the previous study, lower bodyweights and a reduction in food consumption

in F0 males was observed following administration of 2500 ppm thiamethoxam. There was no effect of treatment on the bodyweight or food consumption of the F1 males.

1.2.1 Sperm parameters

[redacted] (2004a) assessed sperm parameters in accordance with the US Guideline (Seed *et al.* 1996) and CTL standard operating procedures ([redacted] 2003a, 2003b; [redacted] 1996), confirming the appropriateness of the methodology. Whilst there was no effect on the number of sperm in the right cauda epididymis of F0 males, a statistically significant increase in the sperm counts of F1 males administered 2500 ppm was observed, which was outside of the historical control range of values. The higher number of sperm in the right cauda epididymis was however considered non-adverse. There was also a decrease in the number of sperm cells in the right testis of F1 males given 50, 1000 or 2500 ppm which was not dose-related, with only one value being outside the contemporaneous control range, and hence was considered not to be treatment-related. Overall, no effects were considered to be of toxicological significance, and the testicular and epididymal sperm counts are considered to reflect normal biological variation.

Table 6: Sperm number (millions) – F1 parent males

	Total number of sperm (million)			
	per testis	**per g testis**	**per cauda epididymis**	**per g cauda**
0	87 ± 22	52 ± 14	153 ± 38	505 ± 94
20	93 ± 23	52 ± 12	153 ± 37	523 ± 136
50	70 ± 19**	42 ± 10**	163 ± 53	532 ± 153
1000	63 ± 16**	36 ± 9**	168 ± 49	546 ± 173
2500	74 ± 18*	43 ± 10**	192 ± 37**	620 ± 122**
CTL/RR0942	85 ± 16	48 ± 8	137 ± 51	479 ± 135
CTL/RR0943	69 ± 18	42 ± 10	170 ± 61	545 ± 170

* Statistically significant difference from control group mean, p<0.05 (Student's t-test, 2-sided)
** Statistically significant difference from control group mean, p<0.01 (Student's t-test, 2-sided)

Table 7: Sperm velocity (µm/s) – F1 parent males

ppm	**Straight line velocity**	**Curvilinear velocity**	**Average path velocity**
0	71.6 ± 5.6	305.0 ± 16.7	123.9 ± 7.5
20	71.8 ± 6.0	297.2 ± 19.5	122.1 ± 7.5
50	72.3 ± 5.2	302.4 ± 17.1	123.1 ± 7.8
1000	71.3 ± 7.4	297.7 ± 21.1	120.4 ± 7.9
2500	68.1 ± 5.4*	291.9 ± 14.7**	117.2 ± 6.1**
CTL/RR0942	64.9	277.9 ± 20.1	112.6 ± 8.6
CTL/RR0943	73.7	306.2 ± 17.1	124.0 ± 8.0

* Statistically significant difference from control group mean, p<0.05 (Student's t-test, 2-sided)
** Statistically significant difference from control group mean, p<0.01 (Student's t-test, 2-sided)

There was no effect of treatment on sperm motility, nor straight line, curvilinear or average path velocities. Whilst F1 males administered 2500 ppm presented significantly lower straight line, curvilinear and average path velocities, these were all well within the historical control range. Morphological examination of the sperm did not reveal any treatment-related abnormalities in either generation. Two males from the 2500 ppm group (animal 117 and 129) were excluded from the analysis on the basis of poor quality sperm samples associated with macroscopic abnormalities in the testis and/or cauda epididymis. Overall, there was no effect of treatment with thiamethoxam on sperm parameters.

1.2.2 Developmental landmarks

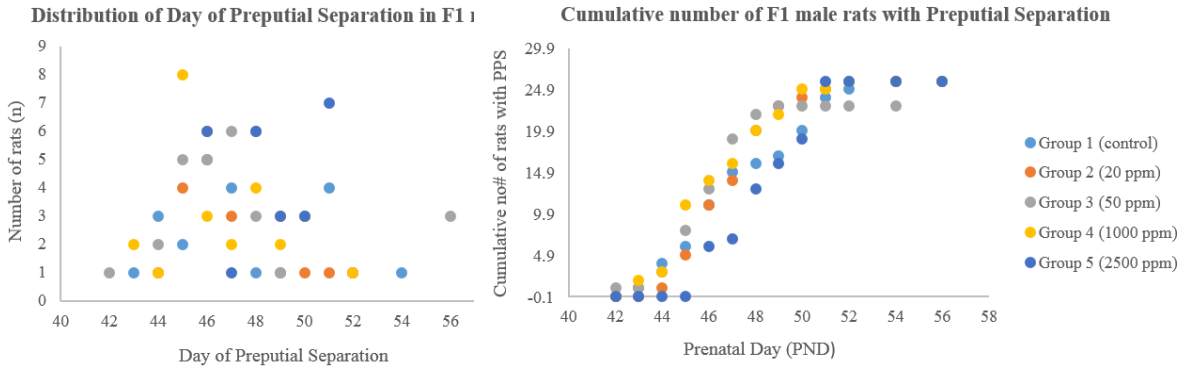
There was no effect of Thiamethoxam on developmental landmarks, such as preputial separation or vaginal opening. However, in conflict with the Study Report, the submitter suggests "*a slight delayed preputial separation (1 day) was observed at the high dose level*" (Table 8).

Table 8: Developmental landmarks F1

	Dose level of Thiamethoxam (ppm)					HCD	HCD
	0	**20**	**50**	**1000**	**2500**	CTL/RR0942	CTL/RR0943
Day of preputial separation	47.7	47.3	47.2	46.7	48.7	47.1	47.2
Standard Deviation (SD)	2.9	2.0	3.6	2.3	1.9		
N	26	26	26	26	26		
BW at preputial separation	173.7	175.8	169.3	170.9	177.7	178.7	181.7

A standard measurement of sexual development, the separation of the prepuce from the glans penis, or balano-preputial separation, is dependent upon bodyweight (Parker 2012; Cameron 1991). In the absence of reductions in bodyweight, a two-day change in the timing of balano-preputial separation in rats is considered to be test item related (Ashby & Lefevre 2000; Clark 1998). All F_1 males acquired balano-preputial separation in the expected time range (PND 43-54 in controls, versus, PND 46-51 in Group 5; Figure 1). The difference in mean values presented in Table 6 is largely attributed to the distribution of data, as reflected in the standard deviation for controls (Table 6). There is no statistically significant, nor toxicologically relevant change in this developmental landmark.

Figure 1. Balano-preputial separation in F1 males ([redacted] 2004a)



1.2.3 Testicular pathology

There was a statistically significantly higher epididymal weight in the 1000 and 2500 ppm F1 males, however this was within the HCD and therefore not considered treatment related. There was a statistically significantly higher testis weight in some of the treated groups, however this was not dose related and therefore considered probably not treatment related.

<u>Table 9: F1 Organ weights (adjusted for final bodyweight) – male endpoints</u>

	Dietary Concentration of Thiamethoxam (ppm)					HCD CTL/RR0942	HCD CTL/RR0943
Organ	**0**	**20**	**50**	**1000**	**2500**		
Epididymis *adj for bw*	1.584	1.617	1.615	1.656*	1.668*	1.572	1.668
Epididymis *absolute*	*1.580*	*1.627*	*1.616*	*1.656*	*1.659**	-	-
Organ to BW ratio	*0.345*	*0.343*	*0.348*	*0.356*	*0.364*	*0.332*	*0.347*
Testes *adj for bw*	3.90	4.12*	4.01	4.13*	4.21**	4.01	4.01
Testes *absolute*	*3.89*	*4.15**	*4.02*	*4.13**	*4.19***	-	-
Organ to BW ratio	*0.85*	*0.87*	*0.87*	*0.87*	*0.92*	*0.85*	*0.83*

* Statistically significant difference from control group mean, p<0.05 (Student's t-test, 2-sided)
** Statistically significant difference from control group mean, p<0.01 (Student's t-test, 2-sided)

The testis, epididymis and efferent ductules were appropriately preserved in Bouin's fixative, trimmed, embedded in paraffin wax, 5µm sections cut and stained with haematoxylin and eosin (H&E). For each testis, four transverse sections were examined and the number of tubular cross sections showing germ cell loss was recorded. Whilst an appropriate methodology was used and the study is considered reliable, it should be noted that the optimal stain for identification of the tubular stages of spermatogenesis was not applied (Lanning *et al.* 2002; OECD GD 16). An increased incidence of very minor, or '*minimal*', testicular lesions was observed in F1 males administered 2500 ppm Thiamethoxam. The increased incidence of germ cell loss/disorganisation +/- Sertoli cell vacuolation observed was considered of low severity with few tubules affected (Table 10). From the satellite group, there was evidence to show that effects were treatment-related since the lesion was bilateral.

Table 10: Incidence of F1 Males with Testicular Tubules showing Germ Cell Loss/Disorganisation +/- Sertoli Cell Vacuolation

	Dose level of Thiamethoxam (ppm)				
	0	**20**	**50**	**1000**	**2500**
Total number of F1 Males (main + satellites)	40 (26+14)	40 (26+14)	40 (26+14)	40 (26+14)	40 (26+14)
Main study: uni/bilateral status unknown	3/26	1/26	1/26	3/26	15/26
Satellites: unilateral	1/14	4/14	2/14	3/14	0/14
Satellites: bilateral	1/14	0/14	0/14	1/14	5/14
Total incidence	5/40	5/40	3/40	7/40	20/40

* Satellite F1 males (n=14/group) were selected on PND 22 and retained to generate histological data on the testis.

The average total number of tubular cross sections affected, in all 4 sections per testis examined from males receiving 2500 ppm was 10.2, which represents approximately 0.4% of tubular cross sections in 4 average testis sections. Significant atrophy or degeneration is likely to manifest as a decrease in organ weight. Consequently, the marginal increase in testicular weight and minimal testicular atrophy (gem cell loss +/- Sertoli cell vacoulation) observed at 2500 ppm do not present a coherent or consistent pathology, and are unlikely to be of toxicological significance.

The fertility and reproductive performance of two generations of parental animals (F0 and F1) was not affected by the administration of Thiamethoxam, nor was there any effect on survival or development. Affecting only small segments of very few tubules, with no effect on reproductive function or spermatogenesis, the minimal histological changes in the testes of F1 males administered 2500 ppm were not considered adverse.

1.3 Adversity of Testicular Effects

In evaluating the intrinsic hazard of a chemical substance, knowledge of the nature and significance of any adverse effect, in addition to the exposure at which this effect is absent or present is key. Whilst frequently used in the scientific literature, terms such as the NOEL, NOAEL and adverse effect are infrequently applied consistently. Consequently, Lewis *et al.* (2002) defined criteria that can be used to make consistent judgements, enabling discrimination between adverse and non-adverse findings. A clear understanding of whether or not treatment-related effects constitute an adverse effect is central to defining hazard.

In this context, adversity is defined as "*a biochemical, morphological or physiological change (in response to a stimulus) that either singly or in combination adversely affects the performance of the whole organism or reduces the organism's ability to respond to an additional environmental challenge*". Thus, functionality, adaptability, transience, severity and coherence are important discriminating factors when differentiating adversity (Lewis *et al*. 2002).

The severity, or grading of atrophy provides an important component of the assessment and a number of different semi-quantitative scoring schemes have been employed (Greaves 2000).

An effect of treatment is less likely to be adverse if the severity of the change is minimal and not related to a change in other parameters (Lewis *et al.* 2002). The testicular atrophy observed ([redacted] 1998, 2000; [redacted] 2004), was minimal to mild, reflecting a histopathological change of limited severity that barely exceeded background variability. Consequently, in the absence of corroborative effects on testicular weight, sperm parameters or reproductive functionality, the testicular atrophy is considered of no toxicological significance.

Table 11: Summary of testicular atrophy across multi-generation studies

[redacted] **(1998; 2000)** **Study: 942121**	[redacted] **(2004)** **Study: RR0941**	**F1 testicular histopathology**
2500 ppm	2500 ppm	In both studies
1000 ppm	1000 ppm	Only in 942121
	50 ppm	None
30 ppm		None
	20 ppm	None
10 ppm		None

The histopathological changes reported involved a very small proportion of the tubules in the affected animals. Detailed histological examination of the remaining unchanged tubules shows that they contain a normal complement of germ cells, and there is no histological evidence of any abnormality of spermatogenesis. Furthermore, there is no correlation between the histological changes and testes weight. In complete contrast to the first study, in the second study testis weights (both absolute and adjusted for bodyweight) were statistically significantly higher than the concurrent control in the F1 generation at 200, 1000, 2500 ppm, but not at 50 ppm. A lack of correlation between the degree of histological change and the testes weights suggest normal variation, rather than an effect of treatment.

In the F1 generation there were statistically significantly lower testicular sperm counts at 50, 1000 and 2500 ppm. However, as the mean values at 50 and 2500 pm are within the contemporaneous control range, this is not considered to be a treatment related effect. In the F1 generation there were statistically significantly higher epididymal sperm counts at 2500 ppm, which are outside the contemporaneous control range. This increase in the numbers of sperm in the right cauda epididymis was not considered to be of toxicological significance. Thiamethoxam has no treatment related effect on sperm production, morphology or motility. Consequently, it can be concluded that the alterations in testicular pathology have no toxicological consequence. There were no treatment related effects seen in either generation, at any dose, on any assessments of reproductive performance and the subtle testicular changes at 1000 and 2500 ppm do not represent a biologically meaningful change. Thus, the findings described for thiamethoxam clearly show:

- ***Microscopic level***: No significant histopathological changes, only an increased incidence of a subtle histopathological change.
- ***Organ level:*** The subtle histopathological change did not alter the structure or function of the testis or related tissues.
- ***Whole animal level:*** No functional consequences. Reproductive performance, sperm number and quality were completely unaffected.

In support of this conclusion, no histopathological changes were noted in the testes of mice and rats in the 90-day studies, nor chronic and carcinogenicity studies ([redacted] 1996; 1998). A decrease in testicular weight was reported at doses >1500 ppm in the 90-day ([redacted] 1996b) and 1-year ([redacted] 1998a) dog studies, with corresponding testicular atrophy at 750 and 1500 ppm in the latter. However, a review of the testicular effects in dogs demonstrated that the lower testicular weight and immature histological appearance were a secondary non-specific consequence of generalised toxicity ([redacted] *et al*. 2019).

1.4 Conclusions

The testicular atrophy observed ([redacted] 1998, 2000; [redacted] 2004), was minimal to mild, reflecting a histopathological change of limited severity that barely exceeded background variability. There subtle histopathological change did not alter the structure or function of the testis or related tissues. There were no functional consequences and reproductive performance, sperm number and quality were completely unaffected. Consequently, in the absence of corroborative effects on testicular weight, sperm parameters or reproductive functionality, the testicular atrophy is considered of no toxicological significance.

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