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TECHNICAL MEMORANDUM

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SUBJECT:	Expert Opinion Regarding Bhide 1990a and Becker 1989 Two-Generation Reproduction Toxicity Studies of Isoproturon

In a previous technical report, Exponent scientists reviewed a published study by Sarkar et al. (1997) reporting male reproductive toxicity due to isoproturon exposure (DeSesso and Williams, 2015). This study was found to suffer from numerous methodological flaws that preclude its use in safety assessment. In particular, the rats used in the study appeared to be undernourished based on both their low initial body weights and minimal weight gains over the course of the 10-week study. In a follow-on to this work, Exponent scientists were asked to review two of the other reproductive toxicity studies that serve as the basis for the proposed European classification of isoproturon as a Category 2 reproductive toxicant. These studies are as follows:

- Bhide, MB. 1990a. Two generation reproduction toxicity study with isoproturon tech administered in the diet to Wistar rats. IIT Project No. 1001.
- Becker, H, Vogel, W, Wilson, JTh, Terrier, Ch. 1989. Isoproturon technical grade (code: Hoe 016410 OH ZD99 0004) – two-generation reproduction study in the rat. RCC Project No. 048846.

The client contacted the laboratory at which the Bhide (1990a) study was conducted; however, the raw data from the study were no longer available. Thus, our analysis is based on the data provided in the study reports only. Our report addresses the shortcomings and deficiencies of the studies. In the case of Bhide (1990a), these deficiencies impact the reliability of the study for its use in reproductive toxicity classification. We also discuss the acceptability of these studies according to the OECD guidelines for conduct of a two-generation reproductive toxicity study, both those established at the time the study was performed as well as the current guideline

requirements. Finally, we provide a comparison of the two studies in terms of their deficiencies and the findings that were reported.

Bhide, 1990a

This reproductive toxicity study was conducted at the Indian Institute of Toxicology (IIT) in Pune, India from 1989 to 1990. In it, groups of male and female Wistar F0 rats were dosed with concentrations of 0, 100, 200 and 400 ppm isoproturon in the diet beginning either 10 weeks (for males) or 2 weeks (for females) prior to mating through generation of the F1 offspring. The F1 offspring, in turn, were described as receiving treatment from birth until sacrifice in adulthood after mating and production of the F2 generation. This means the F1 offspring were exposed in utero during gestation and, presumably, were exposed via milk during lactation (although statements verifying lactational transfer were not in the report). According the European Commission (EC) Proposal for Harmonised Classification and Labelling of Isoproturon (EC, 2015a), this study showed effects of isoproturon on body weights, pregnancy rates, and pup weights, as well as indications of retarded spermatogenesis. However, although this study was reported to have been conducted according to OECD test guideline 416[1] and OECD good laboratory practices (GLP), it suffers from significant reporting deficiencies. Further, as with the study by Sarkar et al. (1997), the available data suggest that the animals were not properly nourished and some of the results reported regarding male reproductive effects are incongruent with those of other, more reliable studies. These weaknesses degrade the reliability of the study for use in hazard assessment.

Significant reporting deficiencies and errors

The study by Bhide (1990a) suffers from substantial reporting deficiencies that make it impossible to reconstruct elements of the study or to properly interpret the data. In particular for assessing effects to the male testes, the methods used to fix and process the tissues for histopathologic examination were not reported. Because the testes are encased in a thick capsule (i.e., tunica albuginea), they should not be immersed whole in fixative. Rather, the tissue should be sectioned or diced prior to fixation to ensure that the fixative fully penetrates the tissue. As an alternative, although less effective, the testes may be pierced in multiple places with a scalpel to assist the fixative in penetrating the tissue. An appropriate fixative that includes acetic acid, such as Bouin's solution or Davidson's solution, is also required to properly fix the testes for histopathologic examination; formalin should not be used. If the testes are not properly processed for examination, tissue autolysis may occur, particularly at the center of the testes. However, because no information is provided regarding the methods for the fixation and processing of tissues, it is not known if the methods were appropriate or if they may have led to possible artifacts due to tissue autolysis.

[1] Note that these guidelines were last updated in 2001, after when the study by Bhide (1990a) was conducted.

Additionally, no historical control data are provided for the laboratory at which the histopathological examination was conducted. The study investigators reported no histopathologic abnormalities in the testes of the parental F0 males, but findings of "retarded spermatogenesis" were noted in 2 of 15 F1 males at the high dose. Further, the study investigators failed to provide a detailed description for the observation of "retarded spermatogenesis." Without this information, we do not know the basis upon which this finding was determined or what the finding means in terms of degree of injury, if any. In addition, the lack of historical background data for this finding in control animals at the conducting laboratory makes it difficult to put this finding in context.

In addition to the lack of methodological detail, no methods for statistical analysis are reported. In fact, from the study report, it appears that no statistical analyses were actually conducted. Therefore, other than subjective reporting of ostensible differences between findings for the control and treatment groups, no conclusions can be drawn regarding whether such findings are statistically significant. The lack of statistical analysis throughout the report is a serious deficiency.

There are other significant reporting deficiencies in this study. For example, no information is provided on how the test article was added to the diet. Consequently, we do not know if the isoproturon was added directly to the feed or first dissolved in water or an organic solvent and, if so, whether the control diet was similarly treated. Such information is relevant as the method of test article incorporation into the diet can affect its bioavailability. Regardless of the initial preparation of the high dose diet, the mid- and low- dose diets were prepared by mixing stock diet to the previously prepared high dose diet and blending it for 2 minutes. It was not reported how (or if) homogeneity of the mid- and low dose diets was verified. We also do not know the mean test article intake levels for the different generations of rats, as this information was not tabulated. Information regarding intake levels is required in current test guidelines and assists in study interpretation. Although the authors discuss body weight changes throughout the study report, no tables of body weight gains are provided. This omission is of particular importance since reduced body weight gains in the F1 parental animals is one of the endpoints used to determine the study's no observed adverse effect level (NOAEL). With regard to reproductive parameters, the methods used to calculate these parameters also were not provided; further, information regarding fertility of the F1 animals is not provided. In addition to these reporting deficiencies, other data were not collected in the study that would have been useful for interpretation of study results. For example, none of the organs were weighed in the study. Such data would have been useful supplementary information for interpretation of the reported histopathologic findings, particularly those described for the testes. Likewise, spermatogenic endpoints (i.e., sperm counts, % abnormal sperm, and motility) were not assessed in the study; this could have been done for both the F0 animals (which were dosed prior to mating for a sufficient period of time to affect all stages of spermatogenesis) and the F1 offspring. Such

information would have been useful in interpreting the qualitative histopathologic effects that were suggested in the testes of the F1 males.

In addition to reporting deficiencies, there are numerous errors within the study report that strongly suggest the investigators were relatively inexperienced with the conduct of reproductive toxicity studies. For example, findings of rough fur and intermittent diarrhea were reported as gross findings for the F0 and F1 animals; however, these types of findings are clinical signs, not gross morphological observations. Additionally, the mating performance for the F0 and F1 generations are summarized in tables VI and XXI, respectively; however, both of these tables have the numbers of sperm positive females and sperm negative females in the wrong table columns. Further, one F1 male at 200 ppm was reported in the text to have retarded spermatogenesis; although this finding was reported in the individual animal tables, it was not tabulated in the summary histopathology table (Table XXXI_1). Finally, the study investigators reported that food consumption was unaffected by treatment; however, examination of the data in Tables XX_1 and XX_2 shows that this was not the case for the F1 males and females. More specifically, the F1 males in the high dose group (400 ppm) consumed approximately 0.5 gram per day less than controls for weeks 5-9 and approximately 1.0 gram per day less than controls in weeks 10-12. Similarly, F1 females in the high dose group (400 ppm) consumed approximately 1.0 gram per day less than controls in weeks 3-12 and up to 2 grams per day less than controls during lactation (data on gestational food consumption were not provided in the study report). These differences from control likely were biologically significant and contribute to the reduced F1 body weight gains and reduced F2 pup weights that were reported at the high dose.

In conclusion, this study suffers from significant reporting deficiencies, omissions, and errors that bring into question the reliability of the study. These issues also make interpretation of the study results for reproductive toxicity classification unreliable.

The use of undernourished animals

This study was conducted with Wistar rats that were reported to be 5 to 6 weeks of age at study initiation. Males were reported to weigh 53-72 grams at study initiation; females were reported to weigh 52-67 grams. The source of rats for this study was the in-house breeding colony of the performing laboratory (IIT). As previously mentioned, historical control data from the laboratory regarding Wistar rats were not provided with the study report and are not readily available. However, growth data for Wistar rats are available from Charles River Laboratories in the United States (Figure 1) and in Germany (Figure 2).

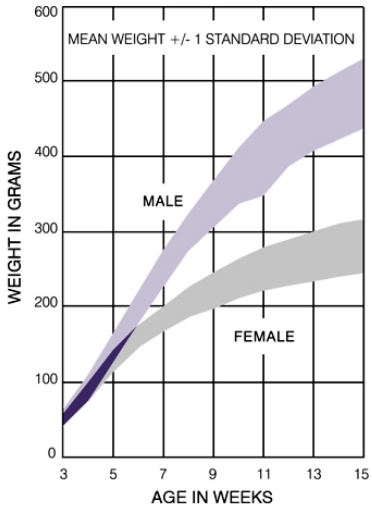

Figure 1. Growth Curve for Charles River Wistar rats in the United States (available at http://www.criver.com/products-services/basic-research/find-a-model/wistar-rat).

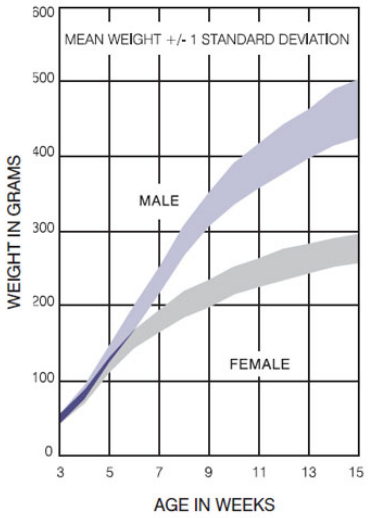

Figure 2. Growth Curve for Charles River Wistar rats in Germany (available at http://www.criver.com/products-services/basic-research/find-a-model/wistar-rat).

As can be seen from these data, male and female Wistar rats typically weigh 50-70 grams around 3 weeks of age. By 5-6 weeks of age, the rats from Charles River laboratories weigh well over 100 grams. Based on this information, the rats used in the Bhide (1990a) study weighed considerably less at study initiation than one would expect.

At the end of 13 weeks (at which time the males would have been approximately 18-19 weeks of age), the mean body weight of control rats in the Bhide (1990a) was only 189 grams. This is in contrast to male Wistar rats from Charles River, which typically weigh over 400 grams by this age. Similarly, the mean body weight of female control rats on the Bhide (1990a) study was 159 grams after 10 weeks on study (at which point they would have been 15-16 weeks of age). Wistar rats from Charles River, in contrast, typically weigh 250-300 grams by this age. These data suggest that the rats used in the study were not of optimal health.

It might be argued that the differences between rats on this study and those shown in Figures 1 and 2 are related to the fact that the study was conducted 25 years ago and the Wistar rat growth curves from Charles River are more contemporary. However, review of EC Proposal for Harmonised Classification and Labelling of Isoproturon (EC, 2015a) shows that rats in other studies conducted around or before the time of the Bhide (1990a) study did not exhibit similarly reduced body weights. Although data for Wistar rats specifically are not available, Leuschner et al. (1973) conducted a 13-week dietary toxicity study in Sprague Dawley rats in 1973 in which

the control male and female rats weighed approximately 411 grams and 253 grams, respectively, by the end of the study (EC, 2015a). Similarly, Wragg et al. (1992) conducted a 13-week dietary study in Sprague-Dawley rats in 1992 in which control male and female rats weighed approximately 537 grams and 313 grams, respectively (EC, 2015a). These data strongly suggest that the reduced weights of the rats used in the Bhide (1990a) study are not related to differences in the strain over time.

Male reproductive effects are incongruent with those of other, more reliable studies

As previously noted, the finding of "retarded spermatogenesis" in the F1 rats was not seen in the F0 generation rats (Bhide, 1990a). Further, review of the EC Proposal for Harmonised Classification and Labelling of Isoproturon (EC, 2015a) shows no evidence of testicular effects in other studies of isoproturon with the exception of the three studies conducted by Bhide (1990a, 1991a, 1991b). These include the only other two-generation reproductive study not conducted at IIT (Becker et al., 1989) in which rats were dosed by dietary administration at concentrations of isoproturon in the feed that overlapped with some of the concentrations administered in the Bhide (1990a) study. In contrast to the Bhide (1990a) study, the study by Becker et al. (1989) was considered *acceptable* for assessing reproductive toxicity according to the Annex B.6 Renewal Assessment Report for Isoproturon (EC, 2015b). Other repeat-dose oral studies summarized in the EC proposal that do not indicate histological changes in the testes include a 28-day mouse dietary study (Hunter et al., 1979); a 28-day oral study in the dog (Kramer and Brunk, 1975); five 90-day oral studies in the rat (Leuschner et al., 1973; Bhide, 1984a; Bhide, 1990b; Dickhaus and Heisler, 1987; Wragg et al., 1992); two 90-day studies in dogs (Scholz and Brunk, 1973; Bhide, 1990c); and a 90-day study in monkeys (Bhide, 1984b). It should be noted that only one of these studies (Wragg et al., 1992) was considered *acceptable*; all others were considered supplementary information because of various deficiencies. Further, while no testicular effects were reported in these studies, based on the EC proposal (EC, 2015a), we only know that the testes were specifically examined in three of these studies (Bhide, 1984a, 1984b; Dickhaus and Heisler, 1987). Nevertheless, other available studies of isoproturon do not report histopathological changes in the testes and do not support the finding of "retarded spermatogenesis."

Acceptability with regard to past and present guidelines

The OECD guidelines provide direction on the accepted procedures, conditions, and reporting of results for the conduct of a two-generation reproductive toxicity testing study. The study by Bhide (1990a) does not fully meet these guidelines, including both those established at the time the study was conducted (OECD, 1983) and the currently adopted guidelines (OECD, 2001). OECD (1983) states that cross-mating procedures should not occur between pups from the same litter. While the study by Bhide (1990a) indicates that "brother-sister matings was avoided *whenever possible*" (emphasis added); whether the matings were exclusively from separate litters is not reported. The OECD (1983) guideline also recommends the evaluation of unsuccessful

mating pairs to determine the cause of the apparent infertility; however, there is no mention of this evaluation having been conducted in the report (Bhide, 1990a). Another evaluation deficiency is in the histopathological analysis. OECD recommends including a detailed description of the microscopic findings, whereas the Bhide (1990a) report merely lists findings without providing additional detail. Further, the methodology used for the histopathological analysis is not described; therefore, interpretation of the reported microscopic changes is unclear. Another shortcoming is that the study report does not mention if a vehicle was used to administer isoproturon in the diet or whether the control rats received vehicle in their diets. The OECD (1983) guideline also suggests performing appropriate statistical analyses on numerical results, which is a fundamental part of any laboratory research study; however, there is no such analysis reported in the Bhide (1990a) study. Even though statistical significance was not reported, the conclusions section nevertheless claims that changes occurred in endpoints such as body weight gains (parental and offspring) and histopathology.

The most current OECD guideline was adopted in 2001 (OECD, 2001) and provides additional, more thorough details on the proper conduct of a two-generation reproductive study. In addition to the deficiencies described above, the study by Bhide (1990a) would be considered *unacceptable* according to current guidelines for several other reasons. For one, additional reproductive functional evaluations are required in the OECD (2001) guideline, including assessment of sperm parameters and estrous cyclicity in all P and F1 animals; these were not reported in the Bhide (1990a) study. The 2001 guideline also suggests weighing several reproductive and non-reproductive organs and examining the uteri for the number of implantation sites as part of the terminal evaluation, but these additional endpoints were not included in the Bhide (1990a) study. Females also were not dosed for several complete estrous cycles prior to mating, as is recommended in the current guideline (OECD, 2001). Instead, isoproturon was administered for two weeks during the pre-mating period, which only provides 2-3 complete estrous cycles. According to the OECD (2001) guideline, offspring selected for mating in the F1 population should be evaluated for sexual maturity (vaginal opening in females and preputial separation in males), and anogenital distance should be determined in F2 pups; the study report by Bhide (1990a) lacks information on these endpoints. The 2001 guideline also recommends histopathologic examination of tissues from at least one weanling per sex per litter from both F1 and F2 generations. This is a new requirement compared to the 1983 OECD guideline, but nonetheless it is noted that this was not included as part of the Bhide (1990a) study. Finally, the Bhide (1990a) study report should have included a rationale for the dose levels, information on stability and homogeneity of the dose preparations, conversion of the concentrations from ppm to mg/kg body weight/day, and details on the food and water quality.

Most importantly, Bhide (1990a) lacks significant detail in the results section and does not provide any statistical analysis of numerical findings, making it *unacceptable* according to both past and current standards. The study is not a reliable predictor of reproductive harm.

Becker, 1989

This reproductive toxicity study was conducted at the Research and Consulting Company (RCC) in Itingen, Switzerland in 1986. Groups of male and female Wistar/Han rats were dosed with concentrations of 0, 80, 400 and 2000 ppm isoproturon in the diet beginning 8 weeks prior to mating through mating, gestation, and lactation. The F1 offspring were described as starting dietary treatment at approximately 4 weeks of age; however, these animals would have been exposed in utero during gestation and, presumably, through the milk during lactation (although information on lactational transfer of isoproturon was not provided). Consequently, the F1 offspring were essentially exposed from prior to conception into adulthood, with the possible exception of a week directly after weaning. The F1 animals were exposed for 17.7 weeks prior to mating, gestation and lactation to produce the F2 generation. According to the EC Proposal for Harmonised Classification and Labelling of Isoproturon (EC, 2015a), this study showed evidence of reduced food consumption and body weights, implantations, litter size, and pup body weights. According to the EC document, the NOEL for purposes of classification was considered to be 80 ppm for both parental and reproductive toxicity. We agree with the NOEL for parental toxicity based on consistent body weight effects at 2000 ppm and occasional but statistically significant body weight effects at 400 ppm. However, the NOEL for reproduction appears to be based on pup weight reductions on postnatal days 14 and 21. These findings are likely due to systemic toxicity as the pups would begin getting into the feed beginning around postnatal day 10. Rather, the NOEL for reproduction conservatively should be 400 ppm based on the reduced litter sizes and some reproductive organ weight effects. The Becker (1989) study was conducted according to GLP and, although not specifically stated in the study report, appears to have been performed according to OECD guidelines for a two-generation reproductive toxicity study, with a few exceptions as stated below. While there are some issues with the study, these issues are relatively minor and do not adversely affect the reliability of the study for use in hazard or safety assessment. The weaknesses in the study are discussed below along with the study's acceptability according to past and present OECD guidelines, followed by a comparison of the findings of the study versus those reported in Bhide (1990a).

Minor study deficiencies

Overall, the study by Becker (1989) was well-conducted with only minor study deficiencies. These deficiencies have minimal impact on the interpretation and reliability of the study; however, for the purposes of review, it is important to be cognizant of these issues and what influence they have on the overall study results.

As with the Bhide (1990a) study, the methods used to fix the testes for histopathologic examination were inadequate. The testes were fixed in 4% formalin solution and no specific measures to ensure penetration of the fixative into the tissues (e.g., sectioning, dicing or piercing of the testes) were reported. As previously noted, formalin is not an optimal fixative for the testes due to its slow penetration of the testis. Improper fixation of the testes can result in tissue autolysis and artifacts that may appear as tissue injury upon histopathologic examination. Nevertheless, it should be noted that the testes were sectioned at 2-4 μm thickness, which is

thinner than necessary for proper histopathologic examination and would allow for greater detection of tissue damage, if present. Importantly, no histopathology of the testes was observed upon examination of the high dose males in either the parental or F1 generations. From the preceding information we can conclude that the methods used for fixation of the testes would bias the results towards the detection of damage. Nevertheless, no such damage was present in treated animals. Further, one control F1 male exhibited atrophy of the testes and hypospermia of the epididymides, confirming that damage to the process of sperm production and maturation was detectable when present. Therefore, although the methods of testes tissue fixation were suboptimal, they do not appear to have adversely affected the reliability of the study results.

It should be further noted that two high dose parental males presented with sperm granulomas in the epididymides; similar findings, however, were not observed in the F1 males. The severity of these findings was graded by the pathologist and they were noted to be both relatively common for Wistar rats and within the expected background incidence, although historical control data for this finding were not provided. Therefore, the sperm granulomas do not appear to be evidence of treatment-related adverse male reproductive effects.

The laboratory's historical control data were provided in the study report; however, these were extremely limited. The data were restricted to reproductive parameters only and appear to have been based on a single study consisting of one control group of 25 parental females and a single group of 25 F1 females. No historical control data on organ weights or histopathology were provided. These deficiencies make interpretation of these types of data more difficult. However, the inclusion of organ weights in the Becker (1989) study (which were not provided in the Bhide, 1990a study) assists in interpretation of the histopathologic findings. With regard to the testes in the parental males, absolute testes weights in the treated groups were not significantly different from control; further, relative weights (compared to body weights) were increased at the high dose compared to control. In the F1 males, absolute testes weight in the high dose group was significantly reduced from control, but relative weights were increased. Additionally, relative testes weights in both the F1 and F2 pups were not significantly different from control. These findings in both the parental and F1 males are not congruent with testicular injury of "retarded spermatogenesis" as reported in Bhide (1990a). For the epididymides, absolute weights were unaffected by treatment; however, the relative weights (compared to body weights) were statistically increased at the high dose in both the parental and F1 adults. Therefore, although historical control data were rather limited for this study, the additional organ weight data are of value in interpreting the histopathologic findings of both the Becker (1989) and Bhide (1990a) studies. While spermatogenic parameters were not assessed in Becker (1989), it should be noted these data were not specifically required according to the OECD guidelines available at the time of the study.

In contrast to the Bhide (1990a) study, statistical analysis of the data in Becker (1989) was conducted. Unfortunately, statistically significant findings were not always identified in the study tables. For example, the statistical significance of food consumption and body weight data

were not shown in the report tables, although they were described in the report text. In contrast, organ weight data that were significantly different from control values were clearly identified in the report tables. Also, it is not known if the reproductive data were appropriately analyzed using the litter as the statistical unit of analysis (Holson and Pearce, 1992; Festing 2006). Typically, if litter data are analyzed based on the fetus, statistical significance is more likely to be found then when it is evaluated using litter means.

In the Becker (1989) study, the only reproductive finding noted to be statistically different from control values was the number of implantations in both the parental and F1 adults in the high dose group. Using ammonium sulfide staining, implantations were reported to be reduced in the high dose group; litter sizes were likewise reduced at the high dose in both generations. Implantations sites are difficult to evaluate in dams that are sacrificed after weaning of their pups. Further, we cannot assess whether this finding is treatment-related because we do not have information regarding the number of corpora lutea. Finally, it should be recognized that the high dose maternal animals in the parental and F1 generations weighed only 89% and 81% of control values, respectively, at the end of the premating period. These reductions in body weights may have contributed to a reduced number of ovulations and thereby resulted in the reduced number of implantations that were observed. Therefore, it is possible that the reduced implantations were a consequence of the systemic toxicity observed at the high dose.

In conclusion, while the study by Becker (1989) presents with some minor issues, these deficiencies have minimal impact on the interpretation and reliability of the study. Further, unlike in the study by Bhide (1990a), no specific data omissions or errors were identified in the Becker (1989) study report. Based on this analysis, it is concluded that the Becker study may be used for reproductive toxicity classification. This conclusion is in line with that of ECHA, which classified the study as “adequate” for the purposes of assessing reproductive toxicity (EC, 2015b).

Acceptability with regard to past and present guidelines

With regard to the OECD (1983) guidelines that were current at the time, the Becker (1989) study had some minor deficiencies. For instance, male rats should have been dosed for at least 70 days prior to mating in order to capture any adverse effects on spermatogenesis; however, male rats were only exposed to isoproturon via the diet for 56 days prior to pairing with females. The 1983 guideline also states that F1 animal dosing should begin at weaning; however, in the Becker et al. (1989) study, treatment of the F1 animals was “considered to have commenced when the selected F1 animals were about 4 weeks of age.” Based on this description, it is unclear whether the F1 animals experienced a disruption in isoproturon exposure for the week after weaning. However, even if a drug holiday occurred, this likely had little effect on the overall study results since the F1 animals were dosed for 124 days prior to mating and few parameters (except body weights and food consumption) were assessed between weaning and reproduction.

In addition to the deficiencies described above, the study by Becker et al. (1989) does not meet some of the requirements of the current testing guidelines (OECD, 2001), but these deficiencies have only a minor impact on interpretation of the study results. The selected dose intervals are greater than the recommended three-fold; however, the selected high dose appears to in accordance with the guideline in that there was no mortality at 2000 ppm. As with Bhide (1990a), some of the additional reproductive functional evaluations required in the OECD (2001) guideline were not reported in the Becker et al. (1989) study; these include assessment of various sperm parameters and estrous cyclicity in the P and F1 animals selected for reproduction, the evaluation of sexual maturity in the F1 animals, and the assessment of anogenital distance in F2 pups. Additionally, a statistical analysis of the number of primordial and growing follicles in ovaries was not conducted. In summary, the Becker (1989) study does not meet a few of the past (OECD, 1983) and current (OECD, 2001) guideline requirements; however, for the most part, these deficiencies are minor and do not affect the overall reliability of the study to predict reproductive harm.

Comparison of Becker (1989) and Bhide (1990a)

Both Becker (1989) and Bhide (1990a) are two-generation reproductive toxicity studies of isoproturon in Wistar rats using the dietary route of exposure. The Becker (1989) study, however, uses a wider range of doses (80-2000 ppm) than the Bhide (1990a) study (100-400 ppm) and presents with fewer and less impactful deficiencies than the study by Bhide (1990a). As previously described, the use of Wistar rats that appeared to be underweight is one major issue that was identified for the Bhide (1990a) study. This does not appear to be as significant a problem for the rats used in the Becker (1989) study. The parental males and females were reported to be 198-255 grams and 137-201 grams, respectively, at the start of test article administration (approximately 8.5 weeks). Compared to the data shown in Figures 1 and 2 of this report, these weights appear to be just within (or slightly lower than) the weight ranges of rats currently available from Charles River Laboratories. At the end of the premating period (approximately 16.5 weeks), control males and females weighed approximately 381 grams and 236 grams, respectively. While the weights of the male rats appear to be outside the current expected ranges according to the grown curves available from Charles River (Figures 1 and 2), the female rats weighed only slightly less than might be expected based on these growth curves.

Significantly, with the exception of testicular toxicity, the findings reported by Bhide (1990a) and Becker (1989) are very similar. Both studies showed effects on food consumption and body weights of both parental animals and pups at doses of 400 ppm or greater. While the Becker (1989) study reported effects on implantations and litter sizes at 2000 ppm, this same dose was not assessed in Bhide (1990a). Furthermore, the reduced implantations are likely due to maternal systemic toxicity. Finally, the testicular histopathologic finding of “retarded spermatogenesis” was not observed in the Becker (1989) study, which was conducted at higher dietary doses and with additional evaluation of organ weights.

Conclusions

As we have described, there are numerous deficiencies and errors in the Bhide (1990a) two-generation reproduction study of isoproturon. These include, but are not limited to, a lack of methodological descriptions for histopathology and calculation of reproductive indices; no statistical analysis of the data; the absence of historical control data for interpretation of study results; a lack of description for reported histopathologic observations; failure to note differences in food consumption rates across treatment groups; and the absence of appropriate tables showing body weight changes over time. Additionally, the animals used in this study appear to have been malnourished or of sub-optimal health, which confounds interpretation of the data. Our overall analysis indicates that the Bhide (1990a) study is *unacceptable* based on both past and current OECD guidelines. While this study has been judged to provide supplementary information for the purposes of classification and labelling (EC, 2015a), we caution that because of its significant deficiencies, errors and omissions, the findings and conclusions of Bhide (1990a) are unreliable and should not be considered as input for identifying reproductive hazards or for use in reproductive toxicity classification.

It should be further noted that two additional reproductive toxicity studies conducted by M.B. Bhide at IIT are also considered by the EC in its proposed classification of isoproturon for reproductive toxicity (EC, 2015a). These additional studies are as follows:

- Bhide, MB. 1991a. Two generation reproduction toxicity study with isoproturon tech administered in the diet to Wistar rats. IIT Project No. 1088.
- Bhide, MB. 1991b. Two generation reproduction toxicity study with isoproturon tech administered in the diet to Wistar rats. IIT Project No. 1096.

These studies were not available to Exponent scientists for review. However, because these two studies were performed at the same contract laboratory, by the same study director, using the same exposure conditions and dietary concentrations, and in the same relative timeframe as the study that is reviewed herein, it is anticipated that many of the criticisms regarding study conduct and reliability also apply to the other two studies from IIT. Similar findings from the additional (nearly identical) Bhide studies should not be considered to be independent verifications of the findings; they merely demonstrate that the laboratory was consistent in performance of the study. Therefore, we urge caution in the way the findings from these additional two studies are interpreted.

The fourth study being considered by the EC in its assessment of isoproturon is the study by Becker (1989). This study appears to have been well-conducted with only minor deficiencies that do not impact the reliability of the study for assessing reproductive hazards. The study included appropriate description of methods, statistical analysis of the data, assessment of both organ weights and histopathology in the P and F1 adult animals as well as in F1 and F2

weanlings, and the use of animals that were of expected age-relevant body weights, and therefore, do not appear to have been compromised with regard the overall health. Further, the range of doses evaluated was greater and the high dose was 5-fold greater than that assessed in the Bhide (1990a) study, allowing for a greater opportunity to observe any treatment-related adverse effects on reproduction, if present. Importantly, the Becker (1989) study does not report adverse findings of the testes, thus calling into question the reliability of the Bhide (1990a) findings.

Because the Bhide studies (1990a, 1991a, 199b) are repetitions of the same study, they should be considered as a single entity that is likely *unacceptable*. Classification of isoproturon for reproductive toxicity should not be based on findings which were observed in these studies alone. Even if the studies were considered as supplementary, the findings from these studies should be used to *supplement* findings reported in more reliable studies. Although all three of the Bhide studies reported "retarded spermatogenesis", evidence of testicular toxicity was not been observed in other, more reliable studies, including subchronic toxicity tests, as already described. Most importantly, no adverse findings of the testes were observed in the Becker (1989) study, which was judged to be adequate. Therefore, without confirmatory support from more dependable and adequate studies, the observations of "retarded spermatogenesis" reported in the Bhide (1990a, 1991a, 1991b) studies should not be considered reliable for the purposes of classification for reproductive toxicity. Consequently, these studies should not be used for reproductive classification of isoproturon, and in particular, for consideration of the fertility endpoint.

In contrast, the Becker (1989) study is considered adequate for the classification of isoproturon for reproductive effects. Our review of the study findings concludes that the reproductive NOEL conservatively appears to be 400 ppm based on a reduction in implantations (which may be due to maternal systemic toxicity) and reduced reproductive organ weights at the high dose of 2000 ppm.

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