Ascorbic acid

Evaluation of the effects on reproduction, recommendation for classification

Gezondheidsraad **Voorzitter**
Health Council of the Netherlands

Aan de minister van Sociale Zaken en Werkgelegenheid

Onderwerp : Aanbieding advies *Ascorbic acid*
Uw kenmerk : DGV/MBO/U-932542
Ons kenmerk : U 5653/HS/pg/543-N11
Bijlagen : 1
Datum : 17 december 2009

Geachte minister,

Graag bied ik u hierbij het advies aan over de effecten van ascorbinezuur op de vruchtbaarheid en het nageslacht; het betreft ook effecten die optreden na blootstelling via de borstvoeding. Dit advies maakt deel uit van een uitgebreide reeks waarin voor de voortplanting giftige stoffen worden geclassificeerd volgens richtlijnen van de Europese Unie. Het gaat om stoffen waaraan mensen tijdens de beroepsuitoefening kunnen worden blootgesteld.

Dit advies is opgesteld door een vaste commissie van de Gezondheidsraad, de Subcommissie Classificatie Reproductietoxische stoffen. Het is vervolgens getoetst door de Beraadsgroep Gezondheid en Omgeving van de raad.

Ik heb dit advies vandaag ook ter kennisname toegezonden aan de minister van Volksgezondheid, Welzijn en Sport en de minister van Volkshuisvesting, Ruimtelijke Ordening en Milieu.

Met vriendelijke groet,

prof. dr. J.A. Knottnerus

Bezoekadres
Parnassusplein 5
2511 VX Den Haag
Telefoon (070) 340 70 04
E-mail: h.stouten@gr.nl

Postadres
Postbus 16052
2500 BB Den Haag
Telefax (070) 340 75 23
www.gr.nl

Ascorbic acid

Evaluation of the effects on reproduction, recommendation for classification

Subcommittee on the Classification of Reproduction Toxic Substances
A Committee of the Health Council of the Netherlands

to:

the Minister of Social Affairs and Employment

No. 2009/03OSH, The Hague, December 17, 2009

The Health Council of the Netherlands, established in 1902, is an independent scientific advisory body. Its remit is “to advise the government and Parliament on the current level of knowledge with respect to public health issues and health (services) research...” (Section 22, Health Act).

The Health Council receives most requests for advice from the Ministers of Health, Welfare & Sport, Housing, Spatial Planning & the Environment, Social Affairs & Employment, Agriculture, Nature & Food Quality, and Education, Culture & Science. The Council can publish advisory reports on its own initiative. It usually does this in order to ask attention for developments or trends that are thought to be relevant to government policy.

Most Health Council reports are prepared by multidisciplinary committees of Dutch or, sometimes, foreign experts, appointed in a personal capacity. The reports are available to the public.

The Health Council of the Netherlands is a member of the European Science Advisory Network for Health (EuSANH), a network of science advisory bodies in Europe.

The Health Council of the Netherlands is a member of the International Network of Agencies for Health Technology Assessment (INAHTA), an international collaboration of organisations engaged with *health technology assessment*.

INAHTA

This report can be downloaded from www.healthcouncil.nl.

Preferred citation:
Health Council of the Netherlands. Ascorbic acid; Evaluation of the effects on reproduction, recommendation for classification. The Hague: Health Council of the Netherlands, 2009; publication no. 2009/03OSH.

all rights reserved

ISBN: 978-90-5549-784-3

Contents

Samenvatting *7*

Executive summary *8*

1 Scope *9*
1.1 Background *9*
1.2 Committee and procedure *9*
1.3 Additional considerations *10*
1.4 Labelling for lactation *11*
1.5 Data *11*
1.6 Presentation of conclusions *12*
1.7 Final remark *12*

2 Ascorbic acid *13*
2.1 Introduction *13*
2.2 Human studies *15*
2.3 Animal studies *16*
2.4 Conclusion *21*

References *24*

Annexes *28*

A The committee *29*

B Directive (93/21/EEC) of the European Community *31*

C Experimental animal fertility and developmental toxicity studies *37*

Samenvatting

Op verzoek van de minister van Sociale Zaken en Werkgelegenheid beoordeelt de Gezondsheidsraad de effecten op de reproductie van stoffen waaraan mensen tijdens de beroepsuitoefening kunnen worden blootgesteld. De Subcommissie Classificatie Reproductietoxische stoffen van de Commissie Gezondheid en Beroepsmatige Blootstelling aan Stoffen (GBBS) van de raad, hierna aangeduid als de commissie, adviseert een classificatie van reproductietoxische stoffen volgens Richtlijn 93/21/EEC van de Europese Unie. Het voorliggende rapport betreft ascorbinezuur.

De aanbevelingen van de commissie zijn:

- voor effecten op de fertiliteit adviseert de commissie om ascorbinezuur niet te classificeren wegens onvoldoende geschikte gegevens.
- voor effecten op de ontwikkeling adviseert de commissie ascorbinezuur niet te classificeren op basis van onvoldoende geschikte humane gegevens en voldoende dierexperimentele gegevens.
- voor effecten tijdens lactatie adviseert de commissie om ascorbinezuur niet te kenmerken wegens onvoldoende geschikte gegevens.

Executive summary

At the request of the Minister of Social Affairs and Employment, the Health Council of the Netherlands evaluates the effects on the reproduction of substances at the workplace. The evaluation and subsequent classification, according to the Directive 93/21/EEC of the European Union, are performed by the Subcommittee on the Classification of Reproduction Toxic Substances of the Dutch Expert Committee on Occupational Safety of the Health Council, hereafter called the committee. In the present report, the committee reviewed ascorbic acid.

The committee's recommendations are:

- for effects on fertility, the committee recommends not classifying ascorbic acid due to a lack of appropriate data.
- for developmental toxicity, the committee recommends not classifying ascorbic acid due to a lack of appropriate human data and due to sufficient animal data.
- for effects during lactation, the committee recommends not labelling ascorbic acid due to a lack of appropriate data.

Chapter 1

Scope

1.1 Background

As a result of the Dutch regulation on registration of compounds toxic to reproduction that came into force on 1 April 1995, the Minister of Social Affairs and Employment requested the Health Council of the Netherlands to classify compounds toxic to reproduction. The classification is performed by the Health Council's Subcommittee on the Classification of Reproduction Toxic Substances, hereafter called the committee, according to the guidelines of the European Union (Directive 93/21/EEC). The committee's advice on the classification will be applied by the Ministry of Social Affairs and Employment to extend the existing list of compounds classified as toxic to reproduction (class 1, 2 or 3) or labelled as 'may cause harm to breastfed babies' (R64).

1.2 Committee and procedure

The present document contains the classification of ascorbic acid by the Health Council's Subcommittee on the Classification of Reproduction Toxic Substances. The members of the committee are listed in Annex A. The classification is based on the evaluation of published human and animal studies concerning adverse effects with respect to fertility and development and lactation of the abovementioned compound.

Classification and labelling was performed according to the guidelines of the European Union listed in Annex B.

Classification for fertility and development:	
Category 1	Substances known to impair fertility in humans (R60)
	Substances known to cause developmental toxicity in humans (R61)
Category 2	Substances which should be regarded as if they impair fertility in humans (R60)
	Substances which should be regarded as if they cause developmental toxicity in humans (R61)
Category 3	Substances which cause concern for human fertility (R62)
	Substances which cause concern for humans owing to possible developmental toxic effects (R63)
No classification for effects on fertility or development	
Labelling for lactation:	
	May cause harm to breastfed babies (R64)
	No labelling for lactation

In 2009, the President of the Health Council released a draft of the report for public review. No comments were received.

1.3 Additional considerations

The classification of compounds toxic to reproduction on the basis of the Directive 93/21/EEC is ultimately dependent on an integrated assessment of the nature of all parental and developmental effects observed, their specificity and adversity, and the dosages at which the various effects occur. The directive necessarily leaves room for interpretation, dependent on the specific data set under consideration. In the process of using the directive, the committee has agreed upon a number of additional considerations.

- If there is sufficient evidence to establish a causal relationship between human exposure to the substance and impaired fertility or subsequent developmental toxic effects in the progeny, the compound will be classified in category 1, irrespective the general toxic effects (see Annex B, 4.2.3.1 category 1).
- Adverse effects in a reproductive or developmental study, in the absence of data on parental toxicity, occurring at dose levels which cause severe toxicity in other studies, need not necessarily lead to a category 2 classification.
- If, after prenatal exposure, small reversible changes in foetal growth and in skeletal development (e.g. wavy ribs, short rib XIII, incomplete ossification) in offspring occur in a higher incidence than in the control group in the

absence of maternal effects, the substance will be classified in category 3 for developmental toxicity. If these effects occur in the presence of maternal toxicity, they will be considered because of this and therefore the substance will not be classified for developmental toxicity (see Annex B, 4.2.3.3 developmental toxicity final paragraph).
- Clear adverse reproductive effects will not be disregarded on the basis of reversibility per se.
- Effects on sex organs in a general toxicity study (e.g. in a subchronic or chronic toxicity study) may warrant classification for fertility.
- The committee not only uses guideline studies (studies performed according to OECD standard protocols* for the classification of compounds, but non-guideline studies are taken into consideration as well.

1.4 Labelling for lactation

The recommendation for labelling substances for effects during lactation is also based on Directive 93/21/EEC. The Directive defines that substances which are absorbed by women and may interfere with lactation or which may be present (including metabolites) in breast milk in amounts sufficient to cause concern for the health of a breastfed child, should be labelled with R64. Unlike the classification of substances for fertility and developmental effects, which is based on a hazard identification only (largely independent of the dosage), the labelling for effects during lactation is based on a risk characterisation and therefore also includes consideration of the level of exposure of the breastfed child.

Consequently, a substance should be labelled for effects during lactation when it is likely that the substance would be present in breast milk in potentially toxic levels. The committee considers a concentration of a compound as potentially toxic to the breastfed child when this concentration is above an exposure limit for the general population, e.g., the acceptable daily intake (ADI).

1.5 Data

Literature searches were conducted in the on-line databases Current Contents and Medline, starting from 1966 up to 2006 and by searches on the internet. An additional search performed in PubMed in May 2009 did not result in relevant additional information. Literature was selected primarily on the basis of the text of the abstracts. Publications cited in the selected articles, but not selected during

* Organisation for Economic Cooperation and Development

the primary search, were reviewed if considered appropriate. In addition, handbooks and a collection of most recent reviews were consulted as well as several websites regarding (publications on) toxicology and health. References are divided in literature cited and literature consulted, but not cited.

Data are described in the text and animal studies with respect to fertility and development are summarised in Annex C. Of each study the quality of the study design (performed according to internationally acknowledged guidelines) and the quality of documentation is considered.

1.6 Presentation of conclusions

The classification is given with key effects, species, and references specified. In case a substance is not classified as toxic to reproduction, one of two reasons is given:

- Lack of appropriate data preclude assessment of the compound for reproductive toxicity.
- Sufficient data show that no classification for toxic to reproduction is indicated.

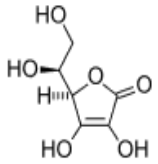
1.7 Final remark

The classification of compounds is based on hazard evaluation (Niesink *et al.*,1995)[1] only, which is one of a series of elements guiding the risk evaluation process. The committee emphasises that for derivation of health-based occupational exposure limits these classifications should be placed in a wider context. For a comprehensive risk evaluation, hazard evaluation should be combined with dose-response assessment, human risk characterisation, human exposure assessment, and recommendations of other organisations.

Chapter 2

Ascorbic acid

2.1 Introduction

name	:	ascorbic acid
CAS number	:	50-81-7
synonyms	:	vitamin C; L-ascorbic acid, L-(+)-ascorbic acid; L-threo-hex-2-enonic acid, gamma lactone; 3-keto-L-gulofuranolactone; L-3-ketothreohexuronic acid lactone; 3-oxo-L-gulofuranolactone; L-xyloasorbic acid; numerous trade names
appearance	:	white to slightly yellow crystals or powder; weakly acidic; water soluble; high melting point
use	:	as a nutrient antioxidant; in vitamin C supplements
molecular formula		$C_6H_8O_6$
structural formula	:	
molecular weight	:	176.12
reference intake	:	In the Netherlands, the recommended dietary allowance is 80 mg/day[2], in agreement with the European Commission directivE.[3]

Ascorbic acid is a naturally occurring substance and is synthesised in most plants and by many animals; e.g., unstressed rats produce about 30-40 mg ascorbic acid/kg bw/day and the average body pool of ascorbic acid amounts to ca. 110 mg/kg bw.[4,5] Ascorbic acid is a strong reducing agent and an antioxidant and as such involved in the prevention of the damaging effects of free radicals. It is a co-factor for enzymes involved in many biochemical reactions, especially those involving oxidations such as the biosynthesis of collagen, neurotransmitters, and carnitine. It also increases the gastrointestinal absorption of non-haem iron by reducing ferric to ferrousion. Primates and guinea pigs are unable to synthesise ascorbic acid endogenously and depend on it in the diet. Ascorbic acid deficiency in humans leads to 'scurvy'. Early symptoms in adults include fatigue, weakness, aching joints and muscles, and, in later stages, anaemia, bleeding gums, petechial and sheet haemorrhages, and delayed wound healing.[5-8]

Ascorbic acid is efficiently absorbed from the gastrointestinal tract (80-90% at daily doses of 30-180 mg) and occurs in the small intestine via a saturable active transport mechanism. Absorption efficiency decreases at higher intakes. Ascorbic acid is widely distributed in all tissues of the body, with higher levels found in the adrenal glands, pituitary, and retina, and lower levels in the kidneys and muscles. Ascorbic acid is metabolised to dehydroascorbic acid which is hydrolised to diketogulonic acid and then oxidised to oxalic acid and threonic acid. At high doses, carbon dioxide can be formed. Unmetabolised ascorbic acid and its metabolites, such as oxalate, are largely excreted in the urine and only small amounts in the faeces (ca. 3% of a 60 mg oral dose). High intakes of ascorbic acid, i.e., above 80-100 mg, result in increasing renal excretion of unchanged ascorbic acid.[6,7]

Experimental animal data indicate that ascorbic acid is of low toxicity. Acute oral LD_{50} values were 8021 mg/kg bw in mice, >5000 mg/kg bw in rats, guinea pigs, and dogs, and >2000 mg/kg bw in rabbits. Also following chronic oral administration, experimental animals appeared to tolerate high doses of ascorbic acid without showing relevant toxic effects.[5] E.g., in a carcinogenicity study in which rats and mice of both sexes received daily amounts of about 2800 and 13,800 mg/kg bw/day, respectively, in their diets for 103 weeks, no compound-related increases in incidences of neoplastic and non-neoplastic lesions were seen.[4] Ascorbic acid was not genotoxic in several bacterial and mammalian test systems, consistent with its anti-oxidant properties. However, in the presence of certain enzyme systems or metal ions, positive results were obtained, consistent with its pro-oxidant activity in these test conditions.[5,6]

In humans, there have been only few controlled studies specifically investigating adverse effects, despite the extensive use of high doses of ascorbic acid in some vitamin supplements. Based on limited data, acute gastrointestinal intolerance (osmotic diarrhoea) is the most clearly defined adverse effect at high intakes.[6-8] No clear causal relationship could be established between excess ascorbic acid intake by apparently healthy individuals and other effects, such as kidney stone formation, excess iron absorption, reduced vitamin B_{12} and copper levels, increased oxygen demand, pro-oxidant effects, and allergic response.[8]

The available data do not allow the establishment of a precise dose at which the gastrointestinal effects appear. However, they suggest that supplemental daily doses up to about 1000 mg in addition to normal dietary intakes are not associated with gastrointestinal effects but that acute gastrointestinal effects may occur at intakes of 3000-4000 mg.

The Scientific Panel on Dietetic Products, Nutrition and Allergies of the European Commission[6] and the UK Expert Group on Vitamins and Minerals[7] concluded that the available data do not allow the establishment of a guidance level or tolerable upper intake level for ascorbic acid. The US Institute of Medicine (IOM) considered that 3000 mg/day is a lowest-observed-adverse-effect level (LOAEL) for gastrointestinal effects and derived a tolerable upper intake level of 2000 mg/day, which holds for adults and pregnant and lactating women. For 0-12-month-old infants, the IOM panel could not derive a tolerable upper intake level because of insufficient data on adverse effects in this age group and concern about the infant's ability to handle excess amounts stemming from isolated reports.[8]

2.2 Human studies

Fertility studies

There are no studies available regarding the effects of exposure to ascorbic acid on human fertility.

Developmental toxicity studies

There are no studies available regarding the effects of exposure to ascorbic acid on development in humans.

Lactation

Several studies showed that in mothers not taking vitamin C supplements, concentrations of ascorbic acid in breast milk ranged from 34 to 83 mg/L while in mothers taking supplements at daily amounts of 45 to >1000 mg, ascorbic acid breast milk levels were between 45 and 115 mg/L. In one of these studies in which maternal ascorbic acid intake ranged from 156 to 1123 mg/day, increased urinary excretion was observed at intakes >200 mg/day.[8]

2.3 Animal studies

In Annex C, fertility and developmental studies performed in animals are summarised.

Fertility studies

Tarin *et al.* (2002)[9] described the effect of dietary administration of ascorbic acid and vitamin E on 'reproductive fitness' of female F1 hybrid (C57Bl/6JIco female x CBA/JIco male) mice. Diet was supplemented with ascorbic acid and vitamin E resulting in average daily intakes of ascorbic acid and vitamin E of 1738-3301 and 109-208 mgkg bw, respectively. A control group was included. Treatment started at the first day of weaning (at 21 days of age). At the age of 28 weeks, females were placed together with 12-week-old male hybrid mice. Pregnant females were killed on gestational day 1 or 12. In the animals sacrificed on gestational day 1, the number of oocytes ovulated and the potential for embryo development *in vitro* to the blastocyst stage (parameters: percentages of total blastocysts, percentage of early, expanding, expanded, and hatching blastocysts) were determined. In the animals sacrificed on gestational day 12, amongst others, the number of corpora lutea and of implantation sites were recorded. No significant effects were seen on the number of zygotes (data from 7 animals), on the blastocyst parameters (data from 7 animals), and on the number of implantation sites (data from 15 animals). However, a lower number of corpora lutea was observed in the left ovaries of treated females when compared to the left ovaries of control mice; the number of corpora lutea in the right ovary was similar to that in control right ovaries (data from 15 animals).

In another series of experiments, 28-week-old females were placed together with 12-week-old males for the rest of their reproductive life during which animals were kept on the same dietary regimen. From day 18 of cohabitation until

the end of the female reproductive life*, females were examined once a day to determine the day of parturition, and litter size and gender of pups at birth in each consecutive litter were recorded. At weaning, offspring was weighted and sacrificed. Treatment did not affect time to pregnancy and age of cessation of female reproductive life.

Effects on maternal animals were not reported.

Developmental toxicity studies

Oral

Basu (1985)[10] studied the conditioning effect of large doses of ascorbic acid in Duncan-Hartley guinea pigs, maintained on a stock diet containing 700 mg ascorbic acid/kg and *ad libitum* water. Two studies were performed. In one study (A), males and females of the test group were given ascorbic acid each day in their drinking water at a concentration of 1 mg/mL from at least 2 weeks before the first mating until the outcome of the second pregnancy. Following birth, the litter was culled to three pups. At weaning, the pups were separated and dams were mated again (not known whether the males were supplemented with ascorbic acid). To study withdrawal effects, the weanling offspring was divided into two groups: one group was continued to be maintained on 1 mg/mL ascorbic acid in drinking water and in the other group, the vitamin exposure in drinking water was reduced to 0.1 mg/mL during 31 days post-weaning. After 31 days, the animals were sacrificed. The control males, females, and weanlings were kept on 0.1 mg/mL ascorbic acid in drinking water throughout the whole study. In the other study (B), the control offspring of study A was supplemented with 1 mg/mL ascorbic acid by drinking water during a period of four weeks after weaning. After this period, animals were divided into two groups: one group continued with the supplementation of 1 mg/mL and in the other group, the supplementation was reduced to 0.1 mg/mL ascorbic acid. After 4 weeks, all animals were sacrificed. At necropsy, in both studies, levels of ascorbic acid in blood and adrenal glands were measured.

The water consumption was not measured, but no apparent difference in intake was observed. Continuous exposure to ascorbic acid (1 mg/mL drinking water) of both males and females from pre-mating up to the outcome of a second pregnancy had no significant effect on the number of offspring and birth weight

* End of reproductive life: age at the last labour following which no more offspring were born for 3 months.

as compared to the control group. The offspring of guinea pigs given 0.1 mg ascorbic acid/mL drinking water throughout pregnancy, lactation, and post-weaning 31 days grew normally. In contrast, exposure to the same amount of ascorbic acid during post-weaning, preceded by a high dose of 1 mg/mL during pregnancy and lactation, resulted in loss of body weight. It appears that the offspring of guinea pigs given high doses throughout pregnancy and lactation had higher requirements for the vitamin. These findings indicate that there may be an adaptation to a high intake of ascorbic acid resulting in a greater than normal metabolism of the vitamin.

Nandi *et al.* (1973)[11] studied the effect of large doses of ascorbic acid given to Charles Foster albino rats. Males (n=6) and females (n=12) were administered 1000 mg ascorbic acid/kg bw/day, dissolved in water and deposited on the back of the tongue using a syringe, for 2 weeks before mating and during mating, and the dams (n=12/group) were subsequently exposed during gestation and lactation. Body weight of females, litter size, and body weight of pups were recorded. Treatment had no effect on maternal body weight (before, during, and after mating; after parturition), litter size, and pup survival and body weights (recorded up to post-natal week 4).

Colomina *et al.* (1994)[12] investigated whether ascorbic acid given to pregnant mice resulted in maternal and developmental toxicity. Swiss mice (number/group unknown) were dosed by gavage with 85 mg ascorbic acid/kg bw on gestational days 6-15. Dams were killed on gestational day 18 and foetuses were examined for external, visceral (soft-tissue), and skeletal abnormalities. The aim of the study was to assess if concurrent ingestion of high doses of aluminium hydroxide and ascorbic acid results in maternal and developmental toxicity. From the results of pregnant mice that only received ascorbic acid during pregnancy, it can be concluded that although the number of implantations per litter was significantly higher than in the control group*, there was no effect on the post-implantation loss, sex ratio, foetal weight, and foetal development.

In a study on the effects of dietary administration of ascorbic acid (1738-3301 mg/kg bw/day) and vitamin E (109-208 mg/kg bw/day) on ‘reproductive fitness’ of female F1 hybrid mice (for study details see above under ‘fertility studies’), Tarin *et al.* (2002)[9] found no effect on number of litters, offspring sex ratio at birth and at weaning, and offspring body weight at weaning in females exposed from the first day of weaning (at 21 days of age) through the end of their reproductive life (at about 87 weeks of age). However, treatment had a negative

* Increased number of implantations is possibly related to stimulation of ovulation (Igarashi, 1977).[23]

effect on frequency of litters, litter size, total number of offspring born, and survival of male pups to weaning. Females sacrificed at day 12 of their first pregnancy had decreased percentages of viable foetuses and higher numbers of resorptions in the left uterine horn when compared to controls' left uterine horns. No statistically significant differences between the control and treated group were seen in the right horns. Foetal weights did not differ between the groups.

Effects on maternal animals were not reported.

Nandi *et al.* (1977)[13] reported the effect of high intake of ascorbic acid by guinea pigs (n=20/group) during pregnancy and lactation on tissue levels of vitamin in the offspring. Approximately 25-day-pregnant guinea pigs were obtained from the supplier and acclimatised to a diet for 5 days. All animals were given 50 mg ascorbic acid/kg bw/day.* On the sixth day, animals were divided into a control group (given 50 mg ascorbic acid/kg bw/day) and an experimental group (given 1000 mg/kg bw/day). During the lactation period, the mothers received the same treatment as during pregnancy. After weaning, the pups were administered doses of ascorbic acid of 10, 20, 30, 40, or 50 mg/kg bw/day for six weeks. In dams and pups, physical appearance, behavioural pattern, body weight gain, and food consumption were measured. In the offspring, tissue contents of ascorbic acid were measured in liver, kidney, brain, and plasma after six weeks of treatment. There was no effect on maternal body weight and food consumption and pup birth weights. An effect on pup weight gain however, was seen in the period after weaning when pups were maintained on graded doses of ascorbic acid for six weeks. It appeared that in pups derived from the experimental group, a diet supplementation of >30 mg/kg was needed to obtain the same weight gain and the same ascorbic acid tissue levels as in pups derived from the control animals. The data presented suggest that the requirement for ascorbic acid in pups after weaning is increased when mothers received large doses during pregnancy and lactation.

Alleva *et al.* (1976)[14] studied the effect of large doses of ascorbic acid on pregnancy in guinea pigs, rats, and hamsters. Fifty-three female guinea pigs were mated. Eleven females were treated twice daily subcutaneously with doses of 400 mg/kg bw/day starting at gestational day 6. Because of inflammatory effects, probably caused by the acidity of the test substance, ascorbic acid was given orally from gestational day 11 onwards until birth. Thirteen controls received saline (probably given subcutaneously during the whole pregnancy period). In the remaining 29 guinea pigs, daily oral treatments with water or ascorbic acid

* All doses of ascorbic acid were deposited as water solutions on the back of the tongue using a syringe.

were initiated after pregnancy was established. Female Holtzman rats were mated with males. Females were dosed orally once a day with doses of 0 (n=14), 50 (n=11), 150 (n=11), or 450 (n=11) mg ascorbic acid/kg bw/day during gestational days 1 to 19. Lakeview hamsters were mated and treated orally with 0 (n=11), 50 (n=12), 150 (n=10), and 450 (n=14) mg ascorbic acid/kg bw/day during gestational days 1 to 16. The study (of poor quality) revealed no adverse effect of large daily doses of ascorbic acid.

Frohberg *et al.* (1973)[15] reported the outcome of reproduction toxicological studies with ascorbic acid in mice and rats. Ascorbic acid was administered to pregnant Wistar rats by gavage at daily doses of 0, 150, 250, 500, and 1000 mg/kg bw from gestational day 6 to 15 (n=17-22/group) and from gestational day 0 to post-natal day 21 (n=24-27/group). Pregnant NMRI mice (n=21-23/group) received doses of 0, 250, 500, and 1000 mg/kg bw/day from gestational day 6 to15. No maternal, foetotoxic or teratogenic effects were seen, and there were no effects on parturition, lactation, and post-partum development of the pups.

Parenteral

In a study on the effects of ascorbic acid on cyclophosphamide-induced embryotoxicity, Pillans *et al.* (1990)[16] treated groups of 15 and 28 pregnant C_3H mice with ascorbic acid only at doses of 3340 and 6680 mg ascorbic acid/kg bw, respectively, injected intraperitoneally on gestational day 11. On gestational day 18, mice were sacrificed and foetal weights, gross morphological malformations, and foetal mortality were recorded. Compared with a control group (n=16), receiving neither ascorbic acid nor cyclophosphamide, no effects were observed in the low-dose group. In the high-dose group, foetal mortality was increased (46% vs. 4%); the surviving foetuses had no gross malformations but higher body weights.

Lactation

Nandi *et al.* (1973)[11] (see above under ‘developmental toxicity studies’) reported that doses of ascorbic acid of 1000 mg/kg bw /day given orally to female Charles Foster albino rats during lactation had no effect on pup survival and body weight (recorded up to post-natal week 4).

Beneficial effects

Literature searches on ascorbic acid and effects on fertility and foetal development resulted in many hits related to beneficial effects. Since this report is focussed on *adverse* effects of compounds, these studies are not described in detail but only mentioned below.

Literature concerning beneficial effects on fertility and prenatal development in women:

- increased foetal birth weight and length (Lee *et al.*, 2004)[17]
- lower risk on preterm delivery (Siega Riz *et al.*, 2003)[18]
- stimulation of ovulation (Igarashi, 1977; Wilson and Loh, 1973)[19,20]

Literature concerning beneficial effects on fertility in man:

- positive association with sperm number (Eskenazi *et al.*, 2005; Sinclair, 2000)[21,22] and pregnancy index (Sinclair, 2000)[22]
- protecting sperm from endogenous oxidative DNA damage (Marik, 2000; Fraga *et al.*, 1991)[23,24]
- decrease in percentage of agglutinated sperm (Gonzalez,1983)[25]
- maintainance of physiological integrity of testis, epididymis, and accessory glands (Dawson *et al.*, 1987)[26]

Literature concerning beneficial effects on fertility and prenatal development in animals:

- increased sperm concentration and plasma testosterone levels (Sönmez *et al.*, 2004)[27]
- associated with reduced formation of multinucleated giant cells (indicative of degeneration) in male turkeys (Neuman *et al.*, 2002)[28]
- protecting sperm from endogenous oxidative DNA damage in teleost fish (Dabrowski and Ciereszko, 1996)[29]
- maintenance of pregnancy in guinea pigs (Habibzadeh *et al.*, 1986; Edwards, 1966)[30,31]

2.4 Conclusion

Fertility

There are no studies available regarding the adverse effects of exposure to ascorbic acid on human fertility.

In animals studies, adverse effects on fertility in mice (reduced number of corpora lutea) were reported after supplementation of diet with high oral doses (>1700 mg/kg/day) ascorbic acid (Tarin *et al.*, 2002).[9] Overall, the committee proposes not classifying ascorbic acid for fertility due to a lack of appropriate data.

Development

There are no studies available regarding the effects of exposure to ascorbic acid on development in humans.

In animal studies, dietary administration of supplementary doses of ascorbic acid (1738-3301 mg/kg bw/day) and vitamin E (109-208 mg/kg bw/day) caused developmental effects (decreased frequency of litters, litter size, total number of offspring born, and survival of male pups to weaning) (Tarin *et al.*, 2002[9]). However, in several other studies with mice, rats, guinea pigs, and hamsters after oral treatment of ascorbic acid at dose levels up to 1000 mg/kg bw/day, no effects on embryonic, foetal, and post-natal development were seen (Nandi *et al.*, 1973[11]; Colomina *et al.*, 1994[12]; Nandi *et al.*, 1977[13]; Alleva *et al.*, 1976[14], Frohberg *et al.*, 1973[15]). After parenteral treatment of very high doses ascorbic acid (6680 mg/kg bw/day), post-implantation loss was observed in mice (Pillans *et al.*, 1990)[16]. Overall, the committee is of the opinion that sufficient animal data show that no classification is indicated for effects on development.

Lactation

Ascorbic acid is excreted in breast milk. The committee notes that ascorbic acid is essential for humans and that they are not able to produce it endogeneously. Thus, the only source of ascorbic acid for exclusively breastfed infants is through lactation. Taking vitamin C supplements does not seem to influence levels of ascorbic acid in breast milk while data available also suggest that mammary tissue may become saturated at ascorbic acid intake levels >200 mg/day. However, no upper tolerable intake level has been established for 0-12-month-old infants. Therefore, the committee proposes not labelling ascorbic acid for effects during lactation due to a lack of appropriate data.

Beneficial

The literature search on ascorbic acid and effects on fertility and foetal development resulted in many hits related to beneficial effects. From these studies (Lee

et al., 2004; Siega Ritz *et al.*, 2003; Igarashi, 1977; Wilson and Loh, 1973; Eskenazi *et al.*, 2005; Sinclair, 2000; Marik, 1999; Fraga *et al.*, 1991; Gonzalez,1983; Dawson *et al.*, 1987; Sönmez *et al.*, 2004; Neuman *et al.*, 2002; Dabrowski and Ciereszko, 1996; Habibzadeh *et al.*, 1986; Edwards, 1966)[17,19-31], it can be concluded that deficiency to ascorbic acid seems to be more harmful to the reproductive cycle than an overdose.

Ascorbic acid is an antioxidant. Antioxidants are chemicals having 'extra', weakly attached electrons which they can donate to a free radical without themselves becoming unstable. As an antioxidant, ascorbic acid can protect membranes, mitochondria, and nuclei against oxidative damages. It is present in high concentrations (as compared to blood plasma) in the epididymal fluid of several species. It is well known that the sperm plasma membrane contains a high amount of unsaturated fatty acids and is therefore particularly susceptible to peroxidative damage. Lipid peroxidation destroys the structure of the lipid matrix in the membranes of spermatozoa resulting in decreased motility.

Proposed classification for fertility

Lack of appropriate data precludes assessment of ascorbic acid for classification for effects on fertility.

Proposed classification for developmental toxicity

Lack of appropriate human data precludes assessment of ascorbic acid for classification for effects on development and sufficient animal data show that no classification for effects on development is indicated.

Proposed labelling for effects during lactation

Lack of appropriate data precludes the assessment of ascorbic acid for labelling for effects during lactation.

References

1 Niesink RJM, de Vries J, Hoolinger MA, editors. Toxicology, Principles and Applications. Boca Raton (FL), USA: CRC Press; 1995.

2 Minister van Volksgezondheid Welzijn en Sport. Besluit van 3 april 2009, houdende wijziging van het Warenwetbesluit Voedingswaarde-informatie levensmiddelen in verband met richtlijn 2008/100/EG. Staatsblad. 2009; nr 182. [cited 2009 Nov 30]. Available from: https://zoek.officielebekendmakingen.nl/stb-2009-182.html.

3 European Commission. Commission Directive 2008/100/EC of 28 October 2008 amending Council Directive 90/496/EEC on nutrition labelling for foodstuffs as regards recommended daily allowances, energy conversion factors and definitions. Official journal of the European Union 2008; 51: L285/9-12. [cited 2009 Jul 29]. Available from: http://eur-lex.europa.eu.

4 Douglas JF, Huff J, Peters AC. No evidence of carcinogenicity for L-ascorbic acid (vitamin C) in rodents. J Toxicol Environ Health 1984; 14(4): 605-609.

5 Elmore AR. Final report of the safety assessment of l-ascorbic acid, calcium ascorbate, magnesium ascorbate, magnesium ascorbyl phosphate, sodium ascorbate, and sodium ascorbyl phosphate as used in cosmetics. Int J Toxicol 2005; 24 Suppl 2: 51-111.

6 European Food Safety Authority (EFSA). Opinion of the Scientific Panel on Dietetic Products, Nutrition and Allergies on a request from the Commission related to the Tolerable Upper Intake Level of Chloride (Request No EFSA-Q-2003-018) (adopted on 28 April 2004). The EFSA Journal (2004) 59,1-21. [cited 2009 jul 29]. Available from: http://www.efsa.europa.eu/.

7 Expert Group on Vitamins and Minerals. Risk assessment Vitamin C. 2003. [cited 2009 Apr 23] Available from: http://www.food.gov.uk/multimedia/pdfs/evm_c.pdf.

8 Institute of Medicine (IOM). Panel on Dietary Antioxidants and Related Compounds. Vitamin C. In: Dietary reference intakes for vitamin C, vitamin E, selenium, and carotenoids: a report of the Panel on Dietary Antioxidants and Related Compounds, Subcommittees on Upper Reference Levels of Nutritients and of Interpretation and Use of Dietary Reference Intakes, and the Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, Food and Nutrition Board. Washington DC, USA: National Academy Press; 2000: 95-185.

9 Tarin JJ, Perez Albala S, Pertusa JF, Cano A. Oral administration of pharmacological doses of vitamins C and E reduces reproductive fitness and impairs the ovarian and uterine functions of female mice. Theriogenology 2002; 57(5): 1539-1550.

10 Basu TK. The conditioning effect of large doses of ascorbic acid in guinea pigs. Can J Physiol Pharmacol 1985; 63(5): 427-430.

11 Nandi BK, Majumder AK, Subramanian N, Chatterjee IB. Effects of large vitamin C in guinea pigs and rats. J Nutr 1973; 103(12): 1688-1695.

12 Colomina MT, Gomez M, Domingo JL, Corbella J. Lack of maternal and developmental toxicity in mice given high doses of aluminium hydroxide and ascorbic acid during gestation. Pharmacol Toxicol 1994; 74(4-5): 236-239.

13 Nandi BK, Majumder AK, Halder K. Effects of high intake of vitamin C by the guinea pigs in pregnancy and lactation on the tissue levels of the vitamin in their offspring. Int J Vitam Nutr Res 1977; 47(2): 200-205.

14 Alleva FR, Alleva JJ, Balazs T. Effect of large daily doses of ascorbic acid on pregnancy in guinea pigs, rats, and hamsters. Toxicol Appl Pharmacol 1976; 35(2): 393-395.

15 Frohberg H, Gleich J, Kieser H. Reproduktionstoxikologische Studien mit Ascorbinsaure an Mausen und Ratten. Arzneimittelforschung 1973; 23(8): 1081-1082.

16 Pillans PI, Ponzi SF, Parker MI. Effects of ascorbic acid on the mouse embryo and on cyclophosphamide-induced cephalic DNA strand breaks in vivo. Arch Toxicol 1990; 64(5): 423-425.

17 Lee BE, Hong YC, Lee KH, Kim YJ, Kim WK, Chang NS, *et al*. Influence of maternal serum levels of vitamins C and E during the second trimester on birth weight and length. Eur J Clin Nutr 2004; 58(10): 1365-1371.

18 Siega Riz AM, Promislow JH, Savitz DA, Thorp JMJ, McDonald T. Vitamin C intake and the risk of preterm delivery. Am J Obstet Gynecol 2003; 189(2): 519-525.

19 Igarashi M. Augmentative effect of ascorbic acid upon induction of human ovulation in clomiphene-ineffective anovulatory women. Int J Fertil 1977; 22(3): 168-173.

20 Wilson CW. Letter: Vitamin C and fertility. Lancet 1973; 2(7833): 859-860.

21 Eskenazi B, Kidd SA, Marks AR, Sloter E, Block G, Wyrobek AJ. Antioxidant intake is associated with semen quality in healthy men. Hum Reprod 2005; 20(4): 1006-1012.

22 Sinclair S. Male infertility: nutritional and environmental considerations. Altern Med Rev 2000; 5(1): 28-38.

23 Marik JJ. Antioxidants and male infertility. Fertil Steril 2000; 73(5): 1065-1066.

24 Fraga CG, Motchnik PA, Shigenaga MK, Helbock HJ, Jacob RA, Ames BN. Ascorbic acid protects against endogenous oxidative DNA damage in human sperm. Proc Natl Acad Sci USA 1991; 88(24): 11003-11006.

25 Gonzalez ER. Sperm swim singly after vitamin C therapy. JAMA 1983; 249(20): 2747, 2751.

26 Dawson EB, Harris WA, Rankin WE, Charpentier LA, McGanity WJ. Effect of ascorbic acid on male fertility. Ann N Y Acad Sci 1987; 498: 312-323.

27 Sonmez M, Turk G, Yuce A. The effect of ascorbic acid supplementation on sperm quality, lipid peroxidation and testosterone levels of male Wistar rats. Theriogenology 2005; 63(7): 2063-2072.

28 Neuman SL, Orban JI, Lin TL, Latour MA, Hester PY. The effect of dietary ascorbic acid on semen traits and testis histology of male turkey breeders. Poult Sci 2002; 81(2): 265-268.

29 Dabrowski K, Ciereszko A. Ascorbic acid protects against male infertility in a teleost fish. Experientia 1996; 52(2): 97-100.

30 Habibzadeh N, Schorah CJ, Smithells RW. The effects of maternal folic acid and vitamin C nutrition in early pregnancy on reproductive performance in the guinea-pig. Br J Nutr 1986; 55(1): 23-35.

31 Edwards MJ. Prenatal loss of foetuses and abortion in guinea-pigs. Nature 1966; 210(32): 223-224.

Literature consulted but not cited

Aafjes JH, van der Vijver JC. 354 mannen met verminderde vruchtbaarheid. Ned Tijdschr Geneeskd 1976; 120(20): 865-873.

Alfin-Slater RB. Vitamins. School of Public Health, University of California, Los Angeles: 91-97

Blom JH, Dabrowski K. Ascorbic acid metabolism in fish: is there a maternal effect on progency? Aquaculture 1996; 147: 215-224.

Briggs MH. Vitamin C and infertility. Lancet 1973; 2(7830) :677-8.

Buettner GR, Schafer FQ. Free radicals, oxidants, and antioxidants. Teratology 2000; 62(4): 234.

Young DJ de, Bantle JA, Fort DJ. Assessment of the developmental toxicity of ascorbic acid, sodium selenate, coumarin, serotonin, and 13-cis retinoic acid using FETAX. Drug Chem Toxicol 1991; 14(1-2): 127-41.

Lamirande E de, Jiang H, Zini A, Kodama H, Gaynon C. Reactive oxygen species and sperm physiology. Rev Reprod 1997; 2(1): 48-54

Diplock AT. Safety of antioxidant vitamins and beta-carotene. Am J Clin Nutr 1995; 62(6 Suppl): 1510S-1516S.

Johnson FC. The antioxidant vitamins. CRC Crit Rev Food Sci Nutr 1979; 11(3): 217-309.

Hankin ME, Cellier KM. Studies of nutrition in pregnancy. V: Ascorbic acid levels of blood and milk in pregnancy and in lactation. Aust N Z J Obstet Gynaecol 1966; 6(2): 153-60.

Hurley WL. Doane RM. Recent developments in the roles of vitamins and minerals in reproduction. J Dairy Sci 1989; 72: 784-804.

Kieser H. Reproduction-toxicologic studies on ascorbic acid in mice and rats. Naunyn Schmiedebergs Arch Pharmacol 1974; 282; suppl 1: R47.

Khan PK, Sinha SP. Impact of higher doses of vitamin C in modulating pesticide genotoxicity. Teratog Carcinog Mutagen 1994; 14(4): 175-181.
Kratzing CC, Kelly JD. Tissue levels of ascorbic acid during rat gestation. Int J Vitam Nutr Res 1982; 52(3): 326-332.
Kratzing CC , Kelly JD, Kratzing JE. Ascorbic acid in fetal rat brain. J Neurochem 1985; 44(5): 1623-1624.
Lamden MP. Dangers of massive vitamin C intake. N Engl J Med 1971; 284(6): 336-337.
Levine M, Rumsey SC, Daruwala R, Park JB, Wang Y. Criteria and recommendations for vitamin C intake. JAMA 1999; 281(15): 1415-1423.
Levine M, Morita K. Ascorbic acid in endocrine systems. Vitam Horm 1985;42:1-64
Malone JI. Vitamin passage across the placenta. Clin Perinatol 1975; 2(2): 295-307.
Nelson MM, Forfar JO. Associations between drugs administered during pregnancy and congenital abnormalities of the fetus. Br Med J 1971; 1(5748): 523-527.
Pintauro SJ, Bergan JG. Effects of ascorbic acid on in vitro steroidogenesis in guinea pigs. J Nutr 1982; 112(3): 584-591.
Raina V, Gurtoo HL. Effects of vitamins A, C, and E on aflatoxin B1-induced mutagenesis in Salmonella typhimurium TA-98 and TA-100. Teratog Carcinog Mutagen 1985; 5(1): 29-40.
Rumbold AR, Maats FH, Crowther CA. Dietary intake of vitamin C and vitamin E and the development of hypertensive disorders of pregnancy. Eur J Obstet Gynecol Reprod Biol 2005; 119(1): 67-71.
Sharma RK, Agarwal A. Role of reactive oxygen species in male infertility. Urology 1996; 48(6): 835-850.
Silló-Seidl G. Der einfluss von Vitamin C auf die Spermienbeweglichkeit. Zentralbl Gynakol 1962 84: 1662-1664.
Thomas MR, Kawamoto J, Sneed SM, Eakin R. The effects of vitamin C, vitamin B6, and vitamin B12 supplementation on the breast milk and maternal status of well-nourished women. Am J Clin Nutr 1979; 32(8): 1679-1685.
Waine C. Vitamin and mineral supplements. Pharm J 2001: 267: 352-354.
Walingo M. Role of vitamin C (Ascorbic acid) on human health - a review. Afr J Food Agric Nutr Develop 2005; 5(1).
Zalani S, Rajalakshmi R, Parekh LJ. Ascorbic acid concentration of human fetal tissues in relation to fetal size and gestational age. Br J Nutr 1989; 61(3): 601-606.

A	The committee
B	Directive (93/21/EEG) of the European Community
C	Fertility and developmental toxicity studies

Annexes

Annex **A**

The committee

- A.H. Piersma, *chairman*
  Professor in reproductive toxicology, University of Utrecht / National Institute of Public Health and the Environment, Bilthoven
- H.F.P. Joosten
  Toxicologist, formerly NV Organon, Department of Toxicology and Drug Disposition, Oss
- D. Lindhout
  Professor of Medical Genetics, paediatrician, clinical geneticist, University Medical Centre, Utrecht
- N. Roeleveld
  Reproductive epidemiologist, Radboud University Nijmegen Medical Centre, Nijmegen
- J.G. van Vliet
  Reproductive toxicologist, Schering Plough, Oss
- D.H. Waalkens-Berendsen
  Reproductive toxicologist, TNO Quality of Life, Zeist
- P.J.J.M. Weterings
  Toxicologist, Weterings Consultancy BV, Rosmalen
- A.S.A.M. van der Burght, *scientific secretary*
  Health Council of the Netherlands, Den Haag
- J.T.J. Stouten, *scientific secretary*
  Health Council of the Netherlands, Den Haag

The first draft of the present document was prepared by M.M. Tegelenbosch-Schouten (TNO Quality of Life, Zeist, the Netherlands).

The Health Council and interests

Members of Health Council Committees – which also include the members of the Advisory Council on Health Research (RGO) since 1 February 2008 – are appointed in a personal capacity because of their special expertise in the matters to be addressed. Nonetheless, it is precisely because of this expertise that they may also have interests. This in itself does not necessarily present an obstacle for membership of a Health Council Committee. Transparency regarding possible conflicts of interest is nonetheless important, both for the President and members of a Committee and for the President of the Health Council. On being invited to join a Committee, members are asked to submit a form detailing the functions they hold and any other material and immaterial interests which could be relevant for the Committee's work. It is the responsibility of the President of the Health Council to assess whether the interests indicated constitute grounds for non-appointment. An advisorship will then sometimes make it possible to exploit the expertise of the specialist involved. During the establishment meeting the declarations issued are discussed, so that all members of the Committee are aware of each other's possible interests.

Annex **B**

Directive (93/21/EEC) of the European Community

4.2.3 Substances toxic to reproduction

4.2.3.1 For the purposes of classification and labelling and having regard to the present state of knowledge, such substances are divided into 3 categories:

Category 1:

Substances known to impair fertility in humans

There is sufficient evidence to establish a causal relationship between human exposure to the substance and impaired fertility.

Substances known to cause developmental toxicity in humans

There is sufficient evidence to establish a causal relationship between human exposure to the substance and subsequent developmental toxic effects in the progeny.

Category 2:

Substances which should be regarded as if they impair fertility in humans:

There is sufficient evidence to provide a strong presumption that human exposure to the substance may result in impaired fertility on the basis of:

- Clear evidence in animal studies of impaired fertility in the absence of toxic effects, or, evidence of impaired fertility occurring at around the same dose levels as other toxic effects but which is not a secondary non-specific consequence of the other toxic effects.
- Other relevant information.

Substances which should be regarded if they cause developmental toxicity to humans:

There is sufficient evidence to provide a strong presumption that human exposure to the substance may result in developmental toxicity, generally on the basis of:

- Clear results in appropriate animal studies where effects have been observed in the absence of signs of marked maternal toxicity, or at around the same dose levels as other toxic effects but which are not a secondary non-specific consequence of the other toxic effects.
- Other relevant information.

Category 3:

Substances which cause concern for human fertility:

Generally on the basis of:

- Results in appropriate animal studies which provide sufficient evidence to cause a strong suspicion of impaired fertility in the absence of toxic effects, or evidence of impaired fertility occurring at around the same dose levels as other toxic effects, but which is not a secondary non-specific consequence of the other toxic effects, but where the evidence is insufficient to place the substance in Category 2.
- Other relevant information.

Substances which cause concern for humans owing to possible developmental toxic effects:

Generally on the basis of:

- Results in appropriate animal studies which provide sufficient evidence to cause a strong suspicion of developmental toxicity in the absence of signs of marked maternal toxicity, or at around the same dose levels as other toxic effects but which are not a secondary non-specific conse-

quence of the other toxic effects, but where the evidence is insufficient to place the substance in Category 2.

- Other relevant information.

4.2.3.2 The following symbols and specific risk phrases apply:

Category 1:

For substances that impair fertility in humans:
T; R60: May impair fertility

For substances that cause developmental toxicity:
T; R61: May cause harm to the unborn child

Category 2:

For substances that should be regarded as if they impair fertility in humans:
T; R60: May impair fertility

For substances that should be regarded as if they cause developmental toxicity in humans:
T; R61: May cause harm to the unborn child.

Category 3:

For substances which cause concern for human fertility:
Xn; R62: Possible risk of impaired fertility

For substances which cause concern for humans owing to possible developmental toxic effects:
Xn; R63: Possible risk of harm to the unborn child.

4.2.3.3 Comments regarding the categorisation of substances toxic to reproduction

Reproductive toxicity includes impairment of male and female reproductive functions or capacity and the induction of non-inheritable harmful effects on the progeny. This may be classified under two main headings of 1) Effects on male or female fertility, 2) Developmental toxicity.

1 *Effects on male or female fertility,* includes adverse effects on libido, sexual behaviour, any aspect of spermatogenesis or oogenesis, or on hormonal activity or physiological response which would interfere with the capacity to fertilise, fertilisation itself or the development of the fertilised ovum up to and including implantation.

2 *Developmental toxicity*, is taken in its widest sense to include any effect interfering with normal development, both before and after birth. It includes effects induced or manifested prenatally as well as those manifested postnatally. This includes embrytoxic/fetotoxic effects such as reduced body weight, growth and developmental retardation, organ toxicity, death, abortion, structural defects (teratogenic effects), functional defects, peri-postnatal defects, and impaired postnatal mental or physical development up to and including normal pubertal development.

Classification of chemicals as toxic to reproduction is intended to be used for chemicals which have an intrinsic or specific property to produce such toxic effects. Chemicals should not be classified as toxic to reproduction where such effects are solely produced as a non-specific secondary consequence of other toxic effects. Chemicals of most concern are those which are toxic to reproduction at exposure levels which do not produce other signs of toxicity.

The placing of a compound in Category 1 for effects on Fertility and/or Developmental Toxicity is done on the basis of epidemiological data. Placing into Categories 2 or 3 is done primarily on the basis of animal data. Data from *in vitro* studies, or studies on avian eggs, are regarded as 'supportive evidence' and would only exceptionally lead to classification in the absence of *in vivo* data.

In common with most other types of toxic effect, substances demonstrating reproductive toxicity will be expected to have a threshold below which adverse effects would not be demonstrated. Even when clear effects have been demonstrated in animal studies the relevance for humans may be doubtful because of the doses administrated, for example, where effects have been demonstrated only at high doses, or where marked toxicokinetic differences exist, or the route of administration is inappropriate. For these or similar reasons it may be that classification in Category 3, or even no classification, will be warranted.

Annex V of the Directive specifies a limit test in the case of substances of low toxicity. If a dose level of at least 1000 mg/kg orally produces no evidence of effects toxic to reproduction, studies at other dose levels may not be considered necessary. If data are available from studies carried out with doses higher than the above limit dose, this data must be evaluated together with other relevant data. Under normal circumstances it is considered that effects seen only at doses in excess of the limit dose would not necessarily lead to classification as Toxic to Reproduction.

Effects on fertility

For the classification of a substance into Category 2 for impaired fertility, there should normally be clear evidence in one animal species, with supporting evidence on mechanism of action or site of action, or chemical relationship to other known antifertility agents or other information from humans which would lead to the conclusion that effects would be likely to be seen in humans. Where there are

studies in only one species without other relevant supporting evidence then classification in Category 3 may be appropriate.

Since impaired fertility may occur as a non-specific accompaniment to severe generalised toxicity or where there is severe inanition, classification into Category 2 should only be made where there is evidence that there is some degree of specificity of toxicity for the reproductive system. If it was demonstrated that impaired fertility in animal studies was due to failure to mate, then for classification into Category 2, it would normally be necessary to have evidence on the mechanism of action in order to interpret whether any adverse effect such as alteration in pattern of hormonal release would be likely to occur in humans.

Developmental toxicity

For classification into Category 2 there should be clear evidence of adverse effects in well conducted studies in one or more species. Since adverse effects in pregnancy or postnatally may result as a secondary consequence of maternal toxicity, reduced food or water intake, maternal stress, lack of maternal care, specific dietary deficiencies, poor animal husbandry, intercurrent infections, and so on, it is important that the effects observed should occur in well conducted studies and at dose levels which are not associated with marked maternal toxicity. The route of exposure is also important. In particular, the injection of irritant material intraperitoneally may result in local damage to the uterus and its contents, and the results of such studies must be interpreted with caution and on their own would not normally lead to classification.

Classification into Category 3 is based on similar criteria as for Category 2 but may be used where the experimental design has deficiencies which make the conclusions less convincing, or where the possibility that the effects may have been due to non-specific influences such as generalised toxicity cannot be excluded.

In general, classification in category 3 or no category would be assigned on an ad hoc basis where the only effects recorded are small changes in the incidences of spontaneous defects, small changes in the proportions of common variants such as are observed in skeletal examinations, or small differences in postnatal developmental assessments.

Effects during Lactation

Substances which are classified as toxic to reproduction and which also cause concern due to their effects on lactation should in addition be labelled with R64 (see criteria in section 3.2.8).

For the purpose of classification, toxic effects on offspring resulting *only* from exposure via the breast milk, or toxic effects resulting from *direct* exposure of children will not be regarded as 'Toxic to Reproduction', unless such effects result in impaired development of the offspring.

Substances which are not classified as toxic to reproduction but which cause concern due to toxicity when transferred to the baby during the period of lactation should be labelled with R64 (see criteria in section 3.2.8). This R-phrase may also be appropriate for substances which affect the quantity or quality of the milk.

R64 would normally be assigned on the basis of:

a) toxicokinetic studies that would indicate the likelihood that the substance would be present in potentially toxic levels in breast milk, and/or
b) on the basis of results of one or two generation studies in animals which indicate the presence of adverse effects on the offspring due to transfer in the milk, and/or
c) on the basis of evidence in humans indicating a risk to babies during the lactational period. Substances which are known to accumulate in the body and which subsequently may be released into milk during lactation may be labelled with R33 and R64.

Annex **C**

Experimental animal fertility and developmental toxicity studies

Table 1 Fertility studies.

authors	species	experimental period/design	dose /route	general toxicity	effects on reproductive organs/effects on reproduction
Tarin (2002)	F1 hybrid (C57Bl/6JIco female x CBA/JIco male) mouse (n=15/group	male and female mice exposed from first day of weaning onwards during whole reproductive cycle; reproductive performance was recorded	10,000 mg ascorbic acid/kg diet + 630 mg vitamin E/kg diet (i.e., 1738-3301 mg ascorbic acid/kg bw/d; 109-208 mg vit.E/kg bw/d)	not reported	decreased: - frequency of litters during the whole reproductive cycle - litter size - total number of offspring born - survival of the male pups at weaning - number of corpora lutea in the left ovary - percentage of viable foetuses increased: - number of foetal resorptions in the left uterine horn

Table 2 Developmental toxicity studies: oral exposure.

authors	species	experimental period/design	dose/route	general toxicity	effects on reproductive organs/effects on reproduction
Basu (1984)	Hartley guinea pig (n=10/group)	males and females exposed continuously from mating onward to second pregnancy	0.1 mg ascorbic acid/mL drinking water (control), 1 mg ascorbic acid/mL drinking water (treated).	reduced bw in offspring treated with high doses during pregnancy and lactation	no effects
Nandi (1973)	male, female Charles Foster rat (n=12 females; 6 males/group)	males and females exposed for 2 weeks before mating and during mating; pregnant females subsequently during gestation and lactation	1000 mg/kg bw/d; by using a syringe with a 5-cm long 17-gauge blunted needle on the back of the tongue	no effect on maternal bw (before, during, and after mating; after parturition)	no effect on litter size; no effect on pup survival and bw (recorded up to post-natal wk 4)
Colomina (1994)	Swiss mouse (n=unknown)	treatment on GD 6-15. sacrifice on GD 18. reproductive performance assessed, foetuses observed for external, internal, skeletal malformations	85 mg ascorbic acid/kg bw/d; gavage	no maternal toxicity	number of implantations per litter increased; no effect on post-implantation loss, sex ratio, foetal weight, foetal development.
Nandi (1977)	guinea pig	approx. 25-day-pregnant guinea pigs acclimatised to a stock diet for 5 days, plus 50 mg ascorbic acid/kg bw/d by syringe (see above Nandy 1973). On the 6th day, animals divided into control and experimental groups. Dosing continued during lactation. After weaning, pups exposed.	dams: 50 (control), 1000 mg/kg bw/d; pups: 10, 20, 30, 40, 50 mg/kg bw/d, for 6 weeks	no maternal toxicity	bw gain in offspring experimental group reduced
Alleva (1976)	guinea pig (n=11-29/group); Holtzman rat (n=11-14/group); Lakeview hamster (n=10-14/group)	guinea pig: sc (GD 6-10) and oral (GD 11–birth); rat: GD 1-19; hamster: GD 1-16	guinea pig: 400 mg/kg bw/d; rat, hamster: 50, 150, 450 mg/kg bw/d	not reported	no adverse affect (study of poor quality)
Frohberg (1973)	Wistar rat (n=17-22/group; during pregnancy; n=24-27, during pregnancy and lactation); NMRI mouse (n=21-23/group)	rat: GD 6-15 or GD 0 to lactation day 21; mice: GD 6-15	rat: 150, 250, 500,1000 mg/kg bw/d; mice: 250, 500,1000 mg/kg bw/d; gavage	no maternal toxicity	no foetotoxic, teratogenic effects; no effect on parturition, lactation and on post-partum pup development

Table 3 Developmental toxicity studies: parenteral administration.

authors	species	experimental period/design	dose/route	general toxicity	effects on reproductive organs/effects on reproduction
Pillans (1990)	C_3H mice (n=15/group LD n=28/group HD)	single administration on GD 11 (sacrificed on GD 18)	3340, 6680 mg/kg bw; ip	not reported	increased post-implantation loss in the high-dose group; no effect on foetal weight; no abnormalities

n= number of animals; GD= gestation day; LD = low dose; HD = high dose; ip = intraperitoneally; sc = subcutaneously.