Table III Changes in Percentages of CD3+ CD8+ Cytotoxic T cells Expressing Specific Molecules

	Before HIVIg (%)	After HIVIg (%)	*P*-value
CD3+CD8+ cytotoxic T cells			
Perforin	24.5 ± 8.4	28.5 ± 11.0	NS
CD28	77.0 ± 5.5	70.9 ± 9.7	0.05

HIVIg, a high dose of intravenous immunoglobulin therapy (mean ± S.D.)
NS, not significant.

Table IV Changes in Percentages of CD4+CD25+ Regulatory T cells Expressing Specific Molecules

	Before HIVIg (%)	After HIVIg (%)	*P*-value
CD4+CD25+ regulatory T cells			
Foxp3	15.6 ± 8.4	15.7 ± 6.2	NS
CD28	99.2 ± 1.3	99.0 ± 1.2	NS
CD152	14.1 ± 12.3	15.5 ± 8.8	NS
CCR4	51.0 ± 7.6	49.9 ± 7.9	NS

HIVIg, a high dose of intravenous immunoglobulin therapy; (mean ± S.D.).
NS, not significant.

Table V Changes in percentages of CD68+ macrophages expressing specific molecules

	Before HIVIg (%)	After HIVIg (%)	*P*-value
CD68+ macrophages			
CD80	2.8 ± 2.1	2.3 ± 1.5	NS
CD86	77.2 ± 15.3	74.5 ± 15.1	NS
MMP9	24.4 ± 40.2	17.5 ± 30.0	NS
CD206	0.9 ± 1.1	1.1 ± 1.7	NS
CD163	1.6 ± 2.2	1.8 ± 1.9	NS
HLA-DR	77.6 ± 9.4	75.6 ± 13.4	NS
PPAR-γ	9.7 ± 9.3	7.3 ± 6.8	NS
CD36	95.4 ± 5.7	95.2 ± 3.3	NS
CCL22	3.8 ± 4.2	3.8 ± 2.3	NS

HIVIg, a high dose of intravenous immunoglobulin therapy: (mean ± SD).
NS, not significant.

found that expression of inhibitory CD94 on NK cells increased after HIVIg therapy. The expression of inhibitory CD94 was more significantly enhanced by the therapy in six women with live births. Conversely, CD94 expression showed no or little increase in two women with SAAC. The increase in CD94 expression on NK cells resulting from the therapy might be related to desirable fetal prognosis and pharmacodynamic mechanisms of HIVIg efficacy. However, in this study, we experienced no SANC. If we could understand changes in CD94 expression among enough number of women with SANC and SAAC, we would discuss whether an increase in CD94 expression predicted fetal prognosis, or was merely a reflection of HIVIg therapy. It is thought that the activity/cytotoxicity of NK cells results from expression balances of various activating receptors and inhibitory receptors on NK cells.[20–22] Inhibitory

receptor complexes (CD94-NKG2A) belong to the C-type lectin superfamily and contain ITIM sequences in their cytoplasmic tails.[22] HIVIg therapy increases the expression of the inhibitory CD94 receptors and subsequently suppresses NK cell activity/cytotoxicity in the body of RSA women. This is a hypothetical mechanism of possible efficacy of HIVIg therapy for RSA.

It is known that the number of NKT cells increases in the deciduae during early pregnancy;[23] and NKT cells may control the Th cell function at the materno-fetal interface through the production of IFN-γ and IL-4.[24] One report demonstrated that increased number of NKT cells in women with RSA or implantation failure was ameliorated by IVIg therapy, leading to successful pregnancy outcome.[25] In this study, however, NKT cell percentages were not significantly changed by HIVIg therapy.

CD3+CD8+ CTLs may play a role in RSA etiologies. We reported increased expression of perforin on CTLs in the deciduae of women with sporadic SANC when compared with women with sporadic SAAC or women with induced abortions. The expression of perforin on CTLs was inversely correlated with expression of inhibitory CD94 on NK cells in the deciduae.[19] In this study, we measured expression of perforin and CD28 on CTLs. CD28 is a ligand of CD80 and CD86 on antigen presenting cells, and can transduce an activating signal. CD28 expression on CTLs decreased after HIVIg therapy, but with a borderline significance ($P = 0.05$). HIVIg therapy may decrease CD28 expression and subsequently suppress activating signal transduction on CTLs in RSA women. This hypothesis should be further clarified. We measured expression of Treg associated molecules including FOXP3, CD28, CD152 (CTLA-4), and CCR4 in CD4+CD25+ T cells. HIVIg therapy did not cause significant changes in expression percentages of these molecules.

Using a mouse model of immunological reproductive failure, we recently demonstrated that intraperitonial injection of a high dose of immunoglobulin restored the fecundity.[15] Additionally, we found that spleen cells adoptively transferred from immunoglobulin injected donors to recipient mice of reproductive failure restored the fecundity. CD11b+ macrophages transferred from donor mice accumulated selectively in the placentae of recipient mice.[15] Therefore, we expected that macrophages might play a key role in mechanisms of HIVIg efficacy in RSA women. In this study, percentages of macrophages increased after HIVIg therapy, but without statistical significance ($P = 0.06$). Expression of macrophage associated molecules including CD80, CD86, MMP9, CD206, CD163, HLA-DR, PPAR-γ, CD36, or CCL22 in the peripheral blood was not changed by HIVIg therapy. Further investigations are needed.

In this study, we experienced desirable pregnancy outcome, i.e., live births after HIVIg therapy in six patients who had a history of four or more abortions of unexplained etiology. We performed the therapy only once during their early pregnancies; and no additional infusion of immunoglobulin was needed. We believe that HIVIg as immune modifier is effective when this therapy is performed during early pregnancy. Severe RSA cases may have immunologic abnormality as its etiology that can be corrected by HIVIg in early pregnancy.

Acknowledgments

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Outcome of pregnancy in patients with isolated proteinuria

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Purpose of review

The outcome of pregnancy in patients with isolated proteinuria is believed to be favorable. However, whether women with isolated proteinuria are at risk for progressing to preeclampsia has not been extensively studied.

Recent findings

The amount of proteinuria is thought to increase in the early third trimester, irrespective of whether preeclampsia has been diagnosed. A dipstick urinalysis has a poor sensitivity (ranging from 22 to 86%) for the detection of significant proteinuria (≥0.3 g/day). Measurements of the levels of circulating angiogenic factors such as soluble fms-like tyrosine kinase 1, soluble endoglin, vascular endothelial growth factor, and placental growth factor suggest that gestational proteinuria is a mild variant of preeclampsia. In one study, women with isolated proteinuria (≥0.3 g/day) were found to be more likely to progress to preeclampsia than women with isolated hypertension. A considerable number of women with eclampsia exhibited proteinuria alone during their last antenatal visit performed within a week prior to their first convulsion.

Summary

The outcome of women with a retrospective diagnosis of gestational proteinuria is generally favorable. However, a considerable number of women with isolated proteinuria develop hypertension and progress to preeclampsia. Therefore, the statement that the 'outcome of pregnancy in patients with isolated proteinuria is favorable' is misleading. Physicians should be aware of this type of preeclampsia when counseling patients. One possible explanation for the difficulty in diagnosing this form of preeclampsia might be the low sensitivity of the dipstick urinalysis technique for the detection of significant proteinuria.

Keywords

angiogenic factor, antithrombin activity, eclampsia, gestational proteinuria, preeclampsia

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Introduction

The classic clinical presentation of preeclampsia is initial hypertension and subsequent proteinuria. However, whether some pregnant women who initially exhibit proteinuria subsequently develop hypertension has not been extensively studied. The clinical outcomes of such women with gestational proteinuria, defined as women who exhibit transient proteinuria of at least 0.3 g/day appearing at or after 20 weeks of gestation and disappearing by 12 weeks postpartum, are believed to be favorable, and proteinuria is not thought to be independently predictive of an adverse outcome [1].

Outcome of pregnancy in women with isolated proteinuria (gestational proteinuria) diagnosed at 12 weeks postpartum

On the basis of the current criteria adopted in many countries, women with proteinuria alone are not diagnosed as having preeclampsia until they also exhibit hypertension [2,3]; those who have not developed hypertension are diagnosed as having had gestational proteinuria [4]. Thus, gestational proteinuria is a retrospective diagnosis. Indeed, the outcomes of women who exhibit transient proteinuria alone (defined as gestational proteinuria in this article) do not differ largely from the outcomes of healthy controls. Holston *et al.* [5••] observed no significant differences in the duration of pregnancy, the rate of preterm delivery, the incidences of gestational diabetes mellitus and renal dysfunction, the mean birth weight, the rates of small-for-gestational-age infants and perinatal deaths, and the need for neonatal intensive care when 108 women with gestational proteinuria were compared with 1564 controls. In four independent studies [5••,6•,7••,8], the reported numbers of gestational weeks at the time of delivery in women with gestational proteinuria were 36.1 ± 3.3 ($n = 7$), 38.2 ± 1.4 ($n = 18$), 39.4 ± 1.0 ($n = 10$), and 39.4 ± 2.0 ($n = 108$), and the outcomes of the pregnancies were consistently better than

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those of women with preeclampsia. Among these studies, the reported numbers of gestational weeks at the onset of proteinuria were 34.2 ± 2.4 and 34.6 ± 3.8 for women who delivered at gestational week 36.1 ± 3.3 [6•] and 38.2 ± 1.4 [7••], respectively.

What is gestational proteinuria?

Whether gestational proteinuria is a variant of preeclampsia (or a preceding stage of preeclampsia) or merely a sign of physiological alterations in kidney function that are not associated with preeclampsia remains uncertain.

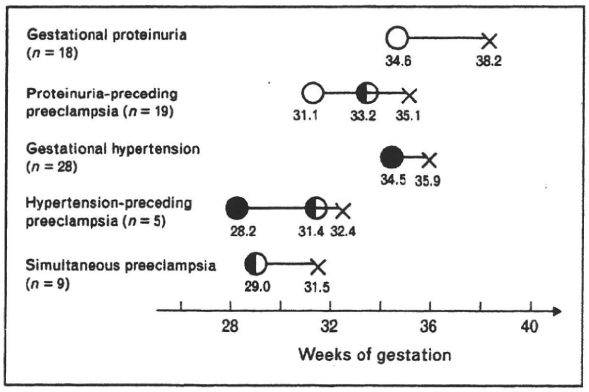
The placental secretion of excessive quantities of antiangiogenic proteins such as soluble fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng) into maternal blood, which causes widespread maternal endothelial dysfunction, is thought to be the final common pathway that leads to preeclampsia [9,10,11•]. Women with preeclampsia have increased serum concentrations of sFlt-1 and sEng and reduced concentrations of free vascular endothelial growth factor (VEGF) and free placental growth factor (PlGF), which are proangiogenic proteins that are bound and neutralized by sFlt-1. Because women with gestational hypertension appear to have similar, but modest, alterations in circulating angiogenic proteins [10], Holston *et al.* [5••] hypothesized that gestational proteinuria might be similar to a mild form of preeclampsia and suggested that it might be accompanied by similar alterations in circulating angiogenic factors. Their hypothesis was subsequently verified: compared with gestational age-matched controls, the PlGF level began to decrease beginning 6–8 weeks before the onset of proteinuria. Although the sFlt-1 and sEng concentrations were elevated 1–2 weeks before the onset of proteinuria, these elevations were modest and transient. Similar findings have also been reported by other groups [6•,8]. The concentrations of PlGF and sFlt-1 in women with gestational proteinuria were intermediate between those in controls and in women with preeclampsia in a study by Masuyama *et al.* [8]. The incidences of a high sFlt-1:PlGF ratio (>95th percentile value), a low PlGF level (<5th percentile value), and a high sEng level (>95th percentile value) in women with gestational proteinuria were 57, 29, and 86%, respectively, whereas those in women with preeclampsia were 94, 77, and 88%, respectively, in a study by Ohkuchi *et al.* [6•]. On the basis of the changes in these predictive parameters of preeclampsia, the authors concluded that gestational proteinuria appears to be a mild variant of preeclampsia or might represent subclinical preeclampsia [5••,6•].

An elevation in the serum urate level occurs in hypertensive pregnancies. However, in a study by Morikawa *et al.* [7••], the reported serum urate level of 5.6 ± 1.5 mg/dl among women with gestational proteinuria tended to be higher than that of 4.8 ± 1.6 mg/dl among women with gestational hypertension but was significantly lower than that of 7.2 ± 1.6 mg/dl among women with preeclampsia [7••]. The incidence of a high serum urate level (>7.0 mg/dl) was 22% for women with gestational proteinuria, 0.0% for women with gestational hypertension, and 67% for women with preeclampsia [7••]. Antithrombin activity is another parameter that is known to be decreased in hypertensive pregnancies [12]. The incidence of a low antithrombin activity (<70%) was 22% for women with gestational proteinuria, 11% for women with gestational hypertension, and 67% for women with preeclampsia [7••]. Although the incidence of a low antithrombin activity of less than 70% among women with uncomplicated pregnancies was not described in the study by Morikawa *et al.* [7••], the rate was less than 3.0% (personal communication). These results may support the notion that gestational proteinuria is a mild variant of preeclampsia or might represent subclinical preeclampsia.

Do women with isolated proteinuria often progress to preeclampsia?

Apparently, some women exhibit transient proteinuria during pregnancy; changes in biological parameters such as the sFlt-1, PlGF, sEng, and uric acid levels and the antithrombin activity, as mentioned above, suggest that these women might have a high risk of developing preeclampsia. Thus, determining whether women with isolated proteinuria actually develop preeclampsia more frequently than women without proteinuria is a concern. If so, the statement that 'the outcome of women with isolated proteinuria is favorable' would be incorrect and misleading from a prospective viewpoint. Although a case report of a woman who initially showed proteinuria and developed hypertension 2 days later has been made [13•], to the best of our knowledge, no case series have dealt with this issue other than a study by Morikawa *et al.* [7••]. They reviewed the medical records of 79 women who developed proteinuria of more than 3.0 g/day and/or hypertension at or after 20 weeks of gestation, focusing on the gestational week at which proteinuria (>0.3 g/day) and/or hypertension developed. Thirty-seven (47%) women exhibited new-onset proteinuria in the absence of hypertension at 32.8 ± 4.8 weeks of gestation, 33 (42%) exhibited new-onset hypertension in the absence of proteinuria at 33.5 ± 4.7 weeks of gestation, and nine (11%) exhibited both proteinuria and hypertension simultaneously at 29.0 ± 4.3 weeks of gestation [7••]. As many as 19 (51%) of the 37 women with isolated proteinuria later developed hypertension at 33.2 ± 4.7 weeks of gestation (Fig. 1), whereas only five (15%) of the 33 women with a presumptive diagnosis of gestational hypertension progressed to preeclampsia ($P = 0.022$). No significant differences in the number of gestational weeks

Figure 1 Mean gestational week at which proteinuria (○), hypertension (●), and delivery (×) developed/occurred in five groups of women



Among 37 women with isolated proteinuria, 19 women who developed hypertension later (proteinuria-preceding preeclampsia) showed proteinuria significantly earlier than the remaining 18 women who remained normotensive (gestational proteinuria). Data from Morikawa *et al.* [7••].

at the time of delivery (36.6 ± 3.4 vs. 35.4 ± 3.5 weeks), the rate of preterm birth at less than 37 weeks (35.1 vs. 42.4%), the rate of fetal growth restriction (21.6 vs. 24.2%), abruptio placenta (10.8 vs. 9.1%), or hemolytic anemia, elevated liver enzymes, and low platelet count (HELLP) syndrome (2.7 vs. 3.3%) were observed between the two starting cohorts of 37 women with isolated proteinuria and the 33 women with a presumptive diagnosis of gestational hypertension. However, in comparison with 19 women diagnosed as having proteinuria preceding preeclampsia and 18 women diagnosed as having gestational proteinuria at 12 weeks postpartum, significant differences were observed in the rate of preterm birth at less than 37 weeks (57.9 vs. 11.1%), birth weight (2142 ± 775 vs. 2797 ± 524 g), antithrombin activity (77.4 ± 13.0 vs. 89.7 ± 18.4%), and the rate of antithrombin activity of less than 70% (42.1 vs. 22.2%). Thus, some women with isolated proteinuria subsequently develop hypertension, and the outcomes of these women who later develop hypertension are worse than that of women with only proteinuria [7••]. Although these results are not obtained from a prospective cohort study, these findings suggest that women with isolated proteinuria may be more likely to progress to preeclampsia than women with a presumptive diagnosis of gestational hypertension, and that approximately half of the women with isolated proteinuria may progress to preeclampsia. The reported rate of progression to preeclampsia, which was 15% (5/33) among women with a presumptive diagnosis of gestational hypertension [7••], was consistent with the result of an earlier study by Saudan *et al.* [14], in which 15–25% of women who were initially diagnosed as having gestational hypertension eventually developed preeclampsia.

Table 1 Appearance of hypertension and proteinuria prior to the first convulsion in women with eclampsia

	1992	2005–2006
Number of patients with eclampsia	325	214
Proteinuria alone	32 (9.8%)	16 (7.5%)
Hypertension alone	71 (21.8%)	20 (9.3%)
Both	186 (57.2%)	81 (37.9%)

The number of women who had hypertension (DBP ≥90 mmHg), proteinuria (≥+1 on dipstick or ≥0.3 g/day), or both during the last antenatal visit within 1 week prior to their first seizure is indicated. Data from Douglas and Redman [15] and Knight *et al.* [16].

Clinicians must be able to counsel pregnant women who develop proteinuria appropriately. The outcome of women with a retrospective diagnosis of 'gestational proteinuria' is indeed favorable, but counseling must be done in a prospective fashion. The statement 'proteinuria is not independently predictive of adverse outcome' [1] is misleading. This conclusion is further supported by the following two studies [15,16]: in the UK, 32 (10%) of 325 women with eclampsia in 1992 and 16 (7.5%) of 214 women with eclampsia between February 2005 and February 2006 exhibited proteinuria alone at the time of their last antenatal visit within 1 week of their first convulsion (Table 1).

Preeclampsia/eclampsia may occur in postpartum women with undiagnosed preeclampsia after hospital discharge [17•]. Yancey *et al.* [17•] reviewed 22 patients who initially presented at an emergency department (ED) during the postpartum period after hospital discharge with complaints such as headache and visual changes: 12 (55%) women had not been diagnosed with preeclampsia in the ante or peripartum period; the time of presentation ranged from 3 to 10 days postpartum, with a median time of 5 days; the initial DBP recorded in the ED ranged from 60 to 114 mmHg, and the DBP was less than 90 mmHg in five (23%) women; 21 (95%) women had either proteinuria, an elevated uric acid level, or an abnormal liver function test.

Thus, hypertension and proteinuria clearly do not necessarily appear together either at the onset of the syndrome or prior to complications, and a considerable number of women with eclampsia have proteinuria alone prior to their first seizure. In addition, it is apparent that women with isolated proteinuria are more likely to develop eclampsia than women with neither proteinuria nor hypertension. Thus, the general belief that 'proteinuria develops late during the course of preeclampsia' might not be correct.

Natural history of new proteinuria in pregnancy and problems with the detection of significant proteinuria

The amount of proteinuria appears to increase during pregnancy. Poon *et al.* [18•] found that the first trimester

median urine albumin concentration and the median albumin-to-creatinine ratio were already significantly elevated in women who developed preeclampsia compared with unaffected individuals. Bar *et al.* [19] examined urinary microalbuminuria levels longitudinally and demonstrated a statistically significant increase in the albumin excretion rate in the second and third trimesters compared with the first trimester. Franceschini *et al.* [20] found that the albumin : creatinine ratio in the urine collected at around 27 weeks of gestation was strongly associated with preterm birth in a dose–response fashion. This association was present for both spontaneous and medically induced preterm birth, and a risk was observable at levels of albuminuria commonly considered to be within the normal range of nonpregnant women and at levels much lower than those detectable using the urine dipstick method, which is commonly used to detect preeclampsia. Gordon *et al.* [21] reported that proteinuria increases from a mean of 1.71 ± 1.33 g/day during the first trimester to a mean of 4.82 ± 4.7 g/day during the third trimester in women with diabetic nephropathy, irrespective of whether the patient is diagnosed as having preeclampsia. Schiff *et al.* [22] reported that proteinuria increases in most women with severe preeclampsia who are managed conservatively (median increase of 660 mg over each 24-h period). Morikawa *et al.* [7••] reported that proteinuria increases with advancing gestation in all three groups of women with gestational proteinuria, proteinuria preceding preeclampsia, or hypertension preceding preeclampsia. These results suggest that protein excretion in the urine begins at a level much lower than 0.3 g/day and continues to increase to at least 0.3 g/day in some women. If this is the case, an appropriate cut-off level for the albumin : creatinine ratio or the protein : creatinine ratio determined at a certain stage of pregnancy might efficiently predict the development of preeclampsia, as has been previously reported [18•,19,20].

Screening for significant proteinuria (≥0.3 g/day) has traditionally been performed using the urine dipstick method, but this method is prone to considerable error. Prior studies [23–27] have reported that a dipstick urinalysis has varying degrees of accuracy, with sensitivities ranging from 22 to 86%. Thus, a considerable number of women with significant proteinuria may have been overlooked and judged not to have proteinuria, leading to the general belief that 'proteinuria develops late in the course of preeclampsia'.

At present, a 24-h urine collection is considered to be the gold standard for quantifying proteinuria. However, a 24-h urine collection is time-consuming and frequently inaccurate, and therefore not a precise measure of proteinuria [28••]. The completeness of a 24-h urine collection should not be assessed by urine volume but rather by urinary creatinine excretion. Twenty-four-hour urinary creatinine excretion reflects muscle mass, and excretion is relatively constant over time in a given person [29]. Although the use of the protein : creatinine ratio to estimate 24-h protein excretion for the diagnosis of preeclampsia has been controversial, two studies [30,31••] describing meta-analyses showed pooled sensitivities of 90 and 84% and specificities of 78 and 76% using various protein : creatinine ratio cut-offs for the detection of significant proteinuria.

Conclusion

Some women with preeclampsia initially exhibit proteinuria followed by the subsequent development of hypertension. Women with isolated proteinuria should be counseled as to the possibility of proteinuria preceding preeclampsia. One possible explanation for the difficulty in diagnosing this type of preeclampsia might be the low sensitivity of the dipstick urinalysis technique. Larger prospective cohort studies using the protein : creatinine ratio and focusing on the gestational week at which proteinuria and/or hypertension develop are needed to determine how often this form of preeclampsia occurs and to search for an efficient protein : creatinine ratio cut-off value for the prediction of adverse outcome.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 543).

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Review

Reproductive and developmental toxicity of degradation products of refrigerants in experimental animals

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ABSTRACT

The present paper summarizes the results of animal studies on the reproductive and developmental toxicity of the degradation products of refrigerants, including trifluoroacetic acid (TFA), carbon dioxide (CO_2), carbon monoxide (CO), carbonyl fluoride (CF), hydrogen fluoride (HF) and formic acid (FA). Excessive CO_2 in the atmosphere is testicular and reproductive toxic, embryolethal, developmentally neurotoxic and teratogenic in experimental animals. As for CO, maternal exposure causes prenatal and postnatal lethality and growth retardation, skeletal variations, cardiomegaly, blood biochemical, immunological and postnatal behavioral changes, and neurological impairment in offspring of several species. Very early studies of CO in rats and guinea pigs reported fetal malformations in exposed dams. The results of toxicological studies on sodium fluoride (NaF) were used to obtain insight into the toxicity of CF and HF, because CF is rapidly hydrolyzed in contact with water yielding CO_2 and HF, and NaF is similar in kinetics and dynamics to HF. Increased fetal skeletal variation, but not malformation, was noted after the maternal administration of NaF. Rat multiple-generation studies revealed that NaF caused retarded ossification and degenerative changes in the lung and kidney in offspring. There is a lack of information about the toxicity of TFA and FA.

Contents

1. Introduction

In 1974, Molina and Rowland noted that chlorofluoromethanes being added to the atmosphere might destroy atmospheric ozone [1]. This was the first startling suggestion that chemical compounds could damage the ozone stratosphere and change the earth's environment. It is now recognized that the rate of destruction has been increased by the presence of harmful ozone-destroying products like fire extinguishers, coolants, foaming agents, solvents, and aerosol propellants created from products like chlorofluorocarbons (CFCs), carbon tetrachloride, bromides, and halons [2]. The Montreal Protocol on Substances that Deplete the Ozone Layer was first established in 1987 and requested that CFCs be phased out prior to the mid-1990s. New refrigerants must not contain chlorine, because it was chlorine which was damaging the ozone layer. New refrigerants must also be efficient because attention was turning to global warming, and the new refrigerants themselves should have little direct global warming [3].

Hydrochlorofluorocarbons (HCFCs) and hydrofluorocarbons (HFCs) were proposed as replacements for ozone-depleting CFCs.

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Table 1
Exposure limits of degradation products of refrigerants.

Compounds	Exposure limits				References
	TLV-TWA[a] (ppm)	TLV-STEL[b] (ppm)	IDLH[c] (ppm)	OEL[d] (ppm)	
Trifluoroacetic acid	–	–	–	–	
Carbon dioxide	5000	30,000	40,000	5000	[21–23]
Carbon monoxide	25	–	1,200	50	[23,30,32]
Carbonyl fluoride	2	5	–	–	[48]
Hydrogen fluoride	0.5 (as F)	2 (as F)	30 (as F)	3	[23,50,51]
Formic acid	5	10	30	5	[23,70,71]

[a] The American Conference of Governmental Industrial Hygienists (ACGIH): threshold limit value-time weighted average concentration (TLV-TWA).
[b] The American Conference of Governmental Industrial Hygienists (ACGIH): threshold limit value-short-term exposure limit (TLV-STEL).
[c] The National Institute of Occupational Safety and Health (NIOSH): Immediately Dangerous To Life or Health Concentration (IDLH).
[d] The Japanese Society for Occupational Health: occupational exposure limit (OEL).

HCFCs still contain chlorine, but their capacity to react with stratospheric ozone is limited. The phasing-out of HCFCs is scheduled by 2020 based on the updated Montreal Protocol. HFCs are being used as acceptable alternatives for CFCs and HCFCs because of low ozone-depleting potential and low global warming. Among HFCs, 1,1,1,2-tetrahydrofluoroethane (HFC-134a) is the most widely used worldwide [4].

Hydroxyl radicals break down HFC-134a to form the CF_3CHFO radical, which reacts with oxygen to generate trifluoroacetyl fluoride (CF_3COF) or undergoes cleavage to give formyl fluoride (HCOF) and the CF_3 radical, which is ultimately converted to carbonyl fluoride (CF: COF_2) and hydrogen fluoride (HF). Acid fluoride, CF_3COF, HCOF and CF, are taken up by clouds and hydrolyzed to trifluoroacetic acid (TFA: CF_3CO_2H), formic acid (FA: CH_2O_2) carbon dioxide (CO_2) and HF [5–7].

In recent years, reproductive and developmental toxicity has increasingly become recognized as an important part of overall toxicology. In fact, adverse effects of environmental chemicals on the reproductive success of wildlife populations have been noted. The possibility of chemicals entering biological systems is of great concern with regard to possible reproductive and developmental toxicity. Although the health effects of refrigerant HFC-134a have been extensively reviewed [4,6–8], the health effects of the products of the degradation of refrigerants are not well understood. In this paper, we mainly summarize the findings of recent animal studies on the reproductive and developmental toxicity of the products of HFC-134a and 2,3,3,3-tetrafluoropropene (HFO-1234yf) developed as a new generation refrigerant [5,9,10].

2. Trifluoroacetic acid (TFA)

TFA is a colorless liquid with a sharp odor produced as a result of industrial activities [11]. TFA is formed from the breakdown of halogenated hydrocarbons such as HFC-134a (CF_3CH_2F), HCFC-124 (CF_3CHFCl) and HCFC-123 (CF_2CHCl_2) [12]. Industrial use of TFA is limited and levels of environmental release are very low. These compounds have only been produced in limited commercial quantities [12]. TFA is a naturally occurring chemical, homogeneously distributed in ocean waters [13]. TFA is not retained in soil, but ultimately enters the aqueous compartment. There are no known pathways for the breakdown of TFA in the environment by either chemical or microbial mechanisms [5].

A threshold limit value-time weighted average concentration (TLV-TWA), threshold limit value-short-term exposure limit (TLV-STEL), immediately dangerous to life or health concentration (IDLH) and occupational exposure limit (OEL) have not yet to be established (Table 1).

TFA is not metabolized in mammalian systems to any great extent [11,14]. Inhalation of, ingestion of, or skin contact with this compound may cause severe injury or death, and contact with the molten substance may cause severe burns to skin and eyes. TFA is a metabolite of the clinically important anesthetic agents isoflurane and halothane, and halothane is developmentally toxic in mice and rats [15] and hepatotoxic in humans and animals [16]. It is also worth noting that halothane readily crosses the placenta and its oxidative metabolite, TFA, accumulates in embryonic and fetal tissues and amniotic fluid in mice and rats [17,18]. In this context, the developmental toxic potential of TFA has been determined. A few reports are available on the reproductive and developmental toxicity of TFA in experimental animals (Table 2). Testicular toxicity was studied in rats. TFA caused no observable testicular effects, including histopathological changes, in Alpk/AP rats given a single oral dose of 10 or 25 mg/kg bw [19]. No effects of TFA were found in an in vitro Sertoli/germ cell co-culture system either. To examine postnatal functional capacities following prenatal exposure to TFA, the offspring of SD rats given TFA by gavage at 75 or 150 mg/kg bw/day on gestational days (GDs) 10–20 were examined on postnatal days (PNDs) 3, 12 and 49 for hepatic and renal biochemistry and/or function through measurements of several serum and urinary parameters [20]. In dams, a significant decrease in body weight gain on GDs 10–15 at 150 mg/kg bw/day and significant increase in the absolute and relative weight of the liver at 75 and 150 mg/kg bw/day were observed. TFA had no adverse effects on the reproductive or developmental parameters. Although the activity of serum glutamate dehydrogenase and aspartate aminotransferase at 75 mg/kg bw/day and higher, and urinary excretion of β_2-microglobulin at 150 mg/kg bw/day were increased in offspring on PND 3, these hepatic and renal changes were not noted on PND

Table 2
Reproductive and developmental toxicity of trifluoroacetic acid in experimental animals.

Animals	Treatment			Findings	References
	Rout	Frequency/length	Dose		
Alpk/AP rat (male)	Gavage	Single	10, 20 mg/kg bw	No adverse effect on testes	[19]
SD rat (female)	Gavage	GDs 10–20	75 mg/kg bw/day	↑Maternal liver wt ↑Serum glutamate dehydrogenase and aspartate aminotransferase in 3-day-old pups ↓Maternal body wt gain ↓Urinary excretion of β_2-microglobulin in 3-day-old pups	[20]

Table 3
Reproductive and developmental toxicity of inhaled carbon dioxide in experimental animals.

Animals	Treatment		Findings	References
	Length/frequency	Concentration		
Swiss mouse (male)	Single 6 h	35%	↓Area/breadth of head/midpiece of spermatozoa in vas deferens	[24]
	4.5 h/day for 6 days	35%	↓Conception rate	
Wistar rat (male)	Single 4 h	2.5%	Reversible degenerative changes in testes	[25]
SD rat (female)	Single 4 h on one of GDs 5–21	6%	↓Viability of offspring, ↑fetal ventricular septal defects	[26]
Rabbit (female)	4–10 h/day for 2–3 days during GDs 7–12	10–13%	↑Fetal skeletal anomalies in vertebral column	[27]
Golden hamster (female)	Single 8 h at 204–212 h after mating	4, 10%	No effect on incidence of embryonic resorption/fetal malformation	[28]
C57BL/6J mouse (female)	Single 8 h on GD 10	20%	↑Maternal plasma PCO_2 ↑serum CO_2 ↓Maternal plasma pH ↑Embryonic resorptions ↑fetal ectrodactyly	[29]
SWV mouse (female)	Single 8 h on GD 10	20%	↑Maternal plasma PCO_2 ↑serum CO_2 ↓Maternal plasma pH	[29]

12 or 49. The authors concluded that prenatal administration of TFA caused transient changes in the liver and kidney of pups during the early postnatal period. Further studies are needed to clarify the toxic effects of TFA on reproduction and development.

3. Carbon dioxide (CO_2)

CO_2 is a colorless, odorless gas categorized as a simple asphyxiant. CO_2 is used in carbonated beverages, fire extinguishers, dry ice and propellants, is a product of animal metabolism and release when organic materials burn, and is a constituent of the earth's atmosphere at about 0.03% by volume [21].

A TLV-TWA of 5000 ppm, TLV-STEL of 30,000 ppm [22], IDLH of 40,000 ppm [21] and OEL of 5000 ppm [23] are recommended for CO_2 (Table 1).

Reproductive and developmental toxicity studies of CO_2 are shown in Table 3. Male mice of the Swiss strain were exposed to a mixture of 65% air and 35% CO_2 by volume for a total of either 6 or 26.5 h (4–4.5 h/day for 6 days) [24]. Exposure for 6 h caused a reduction in the area and breadth of the head and of the midpiece of live spermatozoa in the vas deferens. A decrease in the conception rate, but not in the number of littermates, was found after 26.5 h of exposure, when exposed-males were mated with untreated females. The low conception rate of males appeared to persist even 15 days after the end of exposure to the mixture. The toxicological significance of these findings is equivocal because mice were exposed to a very high concentration of CO_2 and very low concentration of O_2 in this experiment. Male Wistar rats were exposed to an atmosphere containing 2.5%, 5.0%, or 10.0% CO_2 for 1, 2, 4 or 8 h [25]. Exposure to CO_2 at all levels caused a doubling of the respiration rate compared to the control. Typical degenerative changes in the testes were observed after exposure to CO_2 at 2.5% or more for 4 h, and these changes were associated with both the concentration of CO_2 and duration of exposure. The testes had completely recovered to normal histologically 36 h after the exposure. The authors noted that the effects may be due to changes in blood flow or acidosis that occurred with an elevated blood CO_2 concentration and may be entirely indirect.

SD rats were exposed to a gas mixture containing 6% CO_2, 20% O_2 and 74% nitrogen for a single 24-h period on one day of GDs 5–21, and the majority of pregnant rats were allowed to deliver spontaneously [26]. An increased number of rats stillborn or dying shortly after birth and heavier newborn offspring were found in the CO_2-exposed group. The incidence of cardiac malformations was 23% in the CO_2-exposed group and 7% in the control group with the highest incidence occurring when exposure occurred on GD 10. Ventricular septal defects, ventricular septal defects with overriding aorta, and ventricular septal defects with partial transposition were frequently observed. In rabbits, skeletal anomalies in the vertebral column were found in fetuses after exposure to 10–13% CO_2 for 4–10 h on 2 or 3 different days between GDs 7–12 [27]. The value of this study is limited because only three animals were exposed to CO_2. In golden hamsters, no increased incidence of resorptions or malformed fetuses was found in females exposed to 4% or 10% CO_2 for 8 h during 204–212 h after mating [28]. C57BL/6J and SWV mice were exposed to 20% CO_2 for 8 h on GD 10 [29]. Exposure to CO_2 produced marked increases in maternal plasma CO_2 tension (PCO_2) and serum CO_2 accompanied by an inevitable decrease in plasma pH in both strains. Postaxial ectrodactyly was found in 23% of the offspring of C57BL/6J mice, whereas none of the offspring displayed ectrodactytly in SWV mice. The results showed that susceptibility to teratogenesis ranges from the highly sensitive C57BL/6J strain to the resistant SWV strain and there is a correlation between maternal serum CO_2 content and the incidence of ectrodactyly in sensitive strains of mice.

4. Carbon monoxide (CO)

CO is a flammable, colorless, and odorless gas categorized as a chemical asphyxiant. CO is an incomplete combustion product of carbon-containing materials, an emission of internal combustion engines, and also produced by natural processes or by biotransformation of halomethanes within the human body [30]. Motor vehicles are the most important source of CO, and people encounter CO during daily activity such as travelling in motor vehicles, working, cooking or heating with domestic gas, charcoal or wood fires, and inhaling tobacco smoke. Natural background concentrations of CO in remote areas of the southern hemisphere far from human habitation average around 0.05 mg/m^3 (0.04 ppm), primarily as a result of natural processes such as forest fires and the oxidation of methane, which background concentrations in the northern hemisphere are 2–3 times higher because of more extensive human activities [31].

A TLV-TWA of 25 ppm [32], IDLH of 1200 ppm [30], and OEL of 50 ppm [23] are recommended for CO (Table 1).

The health effects of CO are largely the results of the formation of carboxyhemoglobin (COHb), which impairs the oxygen carrying capacity of blood, and the affinity of human hemoglobin for CO is roughly 240 times that for oxygen [31].

Reproductive and developmental toxicity studies are shown in Table 4. In mice, CF-1 females were exposed to an airflow containing CO at 250 ppm for 7 or 24 h daily on GDs 6–15 [33]. The percent COHb plateaued in the range of 10–11% in mice exposed to CO for 7 h daily. A higher incidence of embryonic resorptions, increased fetal body weight, and higher incidence of lumbar spurs were observed after 7-h exposure. After 24-h exposure, a decreased fetal body weight, a shorter fetal crown-rump length, and higher incidences of lumbar ribs and lumbar spurs were detected. CO

Table 4
Reproductive and developmental toxicity of inhaled carbon monoxide in experimental animals.

Animals	Treatment		Findings	References
	Frequency/length	Concentration		
CF-1 mouse (female)	Daily 7 h on GDs 6–15	250 ppm	↑Resorptions ↑fetal wt ↑fetal lumbar spurs	[33]
	Daily 24 h on GDs 6–15	250 ppm	↓Fetal wt ↑fetal lumbar ribs/lumbar spurs	
CD-1 mouse (female)	Continuously on GDs 7–18[a]	125 ppm	↓Fetal wt	[34]
		500 ppm	↑Fetal mortality ↑embryonic/fetal loss	
CD-1 mouse (female)	Continuously on GDs 7–18[a]	65 ppm	↓Mid-air righting reflex	[35]
		125 ppm	↓Righting reflex ↓negative geotaxis	
Wistar rat	Continuously throughout gestation	60 ppm	↑Fetal heart wt	[36]
		125 ppm	↓Fetal wt	
		250 ppm	↓Fetal hemoglobin/hematocrit	
Long–Evans rat (female)	Continuously throughout gestation	150 ppm	↑Absolute/relative wt of heart at 1-day of age ↓Neonatal wt at 1–21 days of age	[37]
Wistar rat (female)	Continuously on GDs 1–22 or 10–22[a] Continuously on GDs 18–22[a]	100 ppm	↓Fetal wt ↑placental wt ↑Placental wt	[38]
SD rat (female)	Daily 2 h throughout gestation	1100–1200 ppm	↓Maternal body wt gain ↓maternal food intake ↑Maternal hematocrit ↓fetal wt ↓placental wt	[39]
Wistar rat (female)	Continuously on GDs 0–20	150 ppm	↓Minimum frequency of ultrasonic calls in 5-day-old male pups ↓Ultrasonic response to diazepam in 10-day-old pups ↓Acquisition of an active avoidance in 3-month-old pups	[40]
Wistar rat (female)	Continuously on GDs 0–20	75 ppm	↓Splenic macrophage killing in 15-day-old male pups	[42]
		150 ppm	↓Splenic macrophage phagocytosis in 15- and 21-day-old male pups	
		150 ppm	↓Splenic macrophage O_2^- release in 15- and 21-day-old male pups	
Wistar rat (female)	Continuously on GDs 0–20	150 ppm	↓Acquisition and reacquisition of active avoidance in 18-month-old male pups	[41]
Rabbit (female)	Daily 24 h on GDs 0–29	90 ppm	↓Neonatal viability ↓Neonatal wt	[43]
NZ rabbit (female)	Daily 24 h on GDs 6–18	250 ppm	↑Fetal crown-rump length	[33]
Miniature/domestic swine (female)	72 h on GDs 108–113	200 ppm	↑Leukoencephalpathy in neonates	[44]
	48–96 h on GDs 108–113	250 ppm	↑Stillbirths	
Crossbred swine (female)	Single 24 h on GD 109	200 ppm	↓Open-field activity at 48 h after birth	[45]
		250 ppm	↓Open-field activity at 24 h after birth ↓Negative geotaxis at 24 h after birth ↑Time to first nursing	
Rhesus monkey (female)	Single 1–3 h at near term pregnancy	0.1–0.3%	↑Newborn brain damage ↑Newborn deaths	[46]

[a] The day on which a vaginal plug/sperm positive vaginal smear was observed was designated as day 1 of pregnancy.

was not found to be teratogenic in mice. CD-1 mice were continuously exposed to 65, 125, 250, or 500 ppm CO in air on GDs 7–18 and sacrificed on GD 18 [34]. No apparent maternal toxicity of CO was observed. Fetal mortality and numbers of dead and resorbed embryos/fetuses were increased at 500 ppm, and fetal body weight was decreased at 125 ppm and higher. No increased incidence of fetal malformations was found after exposure to CO. These findings indicate that developing organisms are sensitive to chronic exposure and lower levels of CO impair growth and higher levels impair viability. CD-1 mice were continuously exposed to 65 or 125 ppm CO in air on GDs 7–18 and allowed to deliver their offspring [35]. CO failed to induce apparent signs of maternal toxicity and adverse effects on numbers of live pups or birth weight. Pups at 125 ppm took longer to complete the righting reflex on PND 1 and negative geotaxis on PND 10. Lower scores of mid-air righting reflex were found in pups at 65 and 125 ppm. It is likely that prenatal exposure to CO at a low concentration impairs reflex development and neuromuscular coordination in neonates. The results of these studies showed that CO during gestation, even at concentrations not toxic to the dam, caused embryo lethality and skeletal variations, decreased growth, and altered in postnatal behavior in offspring.

In rats, Wistar females were continuously exposed to CO at 60, 125, 250, or 500 ppm throughout gestation [36]. Only a slight maternal weight reduction was noted at 500 ppm. Increases in absolute heart weight at 60 and higher and relative heart weight in all four groups exposed to CO were detected. A lower fetal weight was observed at 125 ppm and higher. There was a reduction in fetal hemoglobin and hematocrit levels at 250 ppm and higher. To characterize the change in heart weight after prenatal exposure to CO, biochemical assays and weight measurements were made in neonates of Long–Evans hooded rats exposed to CO at 150 ppm throughout gestation [37]. Levels of COHb were 15% in CO-exposed dams. The offspring exposed to CO weighed less than control offspring at birth, and this weight difference was maintained until PND 21. In the CO-treated group, increases in the absolute and relative wet heart weights, but not dry heart weight, were found in offspring on PND 1. Neither increased heart weight on PNDs 4–21 nor biochemical changes were observed in CO-exposed neonates.

Although cardiomegaly induced by prenatal exposure to CO is likely to be due to edema and transitory, it is not clear whether it alters cardiac function or produce latent cardiovascular effects that may become overt later in life. To determine if periods of exposure would modify the developmental toxicity, female Wistar rats were continuously exposed to CO at 100 ppm throughout gestation, on GDs 1–16, on GDs 4–12, on GDs 10–22 and on GDs 18–22 [38]. Maternal COHb levels were estimated to be in the order of 10–14%. No effects of CO were found on fetal survival. Fetal weight was decreased after exposure to CO on GDs 1–22 and 10–22, and placental weight was increased after exposure to CO on GDs 1–22, 10–22 and 18–22. These results indicate that the rat placenta at near term can become hypertrophic in response to CO, and this response benefits the fetus presumably by improving oxygen transport. SD rats were exposed to CO at 100–1200 ppm daily for 2 h throughout gestation [39]. Lower body weight gain and food intake and a higher hematocrit value were observed in maternal rats after CO exposure. Weights of fetuses and placenta in the CO-exposed group were lower than those of pair-fed and freely fed controls. These data indicate that CO is primarily responsible for the retardation of fetal growth. Wistar rats were continuously exposed to CO at 75 or 15 ppm on GDs 0–20 and allowed to deliver spontaneously [40]. COHb saturation was estimated to be 15% in dams at 150 ppm. There were no adverse effects of prenatal exposure to CO on the dam weight gain, pregnancy length, number of dams giving birth, litter size, or postnatal viability or weight gain of pups. In 14- and 21-day-old pups, open-field activity and D-amphetamine-induced hyperactivity were not affected by prenatal CO exposure. Exposure to CO on GDs 0–20 at 150 ppm caused a reduction in the minimum frequency of ultrasonic calls emitted by PND 5, a decrease in the ultrasonic responsiveness to a dose of diazepam on PND 10, and impairment in the acquisition of an active avoidance schedule in 3-month-old pups. Moreover, both the acquisition and reaquisition of an active avoidance task were markedly impaired in 18-month-old male offspring of dams exposed to CO at 140 ppm throughout pregnancy [41]. These findings showed that prenatal exposure to CO induces both short- and long-term behavioral changes at dose levels below those associated with overt maternal toxicity. Male rat offspring obtained using the same experimental procedure as Di Giovanni et al. [40] were examined for their immunological activity on PNDs 15, 21 and 60 [42]. The phagocytosis of *Candida albicans* and release of O_2^- by splenic macrophages were decreased in offspring on PNDs 15 and 21 at 150 ppm. Prenatal exposure to CO at 75 and 150 ppm reduced the killing by splenic macrophages PND 15. No alterations in the immune system were noted in offspring on PND 60. These results indicate that prenatal exposure to CO caused reversible immunological changes in rat offspring.

In rabbits, New Zealand females were exposed to an airflow containing CO at 250 ppm for 7 or 24 h daily on GDs 6–15 [33]. The percent COHb plateaued in the range of 13–15% in dams exposed to CO for 7 h daily. Fetal weight and crown-rump length were increased after prenatal exposure to CO for 24 h daily. No effects of CO were found on embryonic/fetal viability or morphological development. Rabbits were exposed to air containing CO at 90 or 180 ppm for 30 days, from mating until before expected delivery [43]. Exposure to CO at 90 and 180 ppm produced COHb concentrations of about 8–9% and 16–19%, respectively. The birth-weight decreased approximately 11 and 20% at 90 and 180 ppm, respectively. Within 24 h after birth, about 9.9 and 35% of pups were dead at 90 and 180 ppm, respectively. The mortality of pups during the following 21 days was higher in the 90 ppm group than in the corresponding control group.

In swine, miniature and domestic females were exposed to atmospheric CO at 150, 200, 250, 300, 350 or 400 ppm on GDs 108–113 for 48–96 h [44]. Stillbirths did not occur where the maternal COHb concentrations ranged between 13.8% and 25.8%, but did occur where they ranged between 23.8% and 31.3%. The COHb concentrations in newly delivered pigs were higher than maternal COHb concentrations by 3–22%. Hypoxic ischemic leukoencephalopathy was observed in brain sections from newborn pigs exposed to CO for 72 h at 200 ppm, for 96 h at 300 ppm, or for 48 h at 350 ppm. These findings indicate that near-term exposure to mild concentrations of CO can cause stillbirths and neurotoxicosis in offspring. Crossbred gilts were exposed to atmospheric CO at 200 or 250 ppm for 24 h on GD 109 [45]. The stillbirth rate was only 4.8%. Blood concentrations of COHb were 19.8% at 200 ppm and 22.4% at 250 ppm in neonatal piglets at birth. Piglets at 250 ppm took longer to nurse for the first time. This suggests that the newborn piglet's ability to seek nourishment is hampered, and this has serious implications for piglet survivability. The number of open-field squares entered was decreased in 24-h old piglets at 250 ppm and 48-h old piglets at 200 and 250 ppm. In a negative geotaxis test, the time taken to turn around was longer in 24-h old piglets at 250 ppm. It appears that perinatal exposure to sublethal concentrations of CO has lethal and neurotoxic effects in piglets.

In rhesus monkeys, nine term-pregnant females were exposed to 0.1–0.3% inspired CO over 1–3 h during near term pregnancy [46]. Eight females with dated pregnancies were studied on GDs 156–159, and one female with an undated pregnancy was judged to be near term by maternal abdominal palpation. The COHb levels rose rapidly to approximately 26–62% in dams and gradually to approximately 8–33% in fetuses. No clinical sequelae were observed in dams exposed to CO. Neurological impairments were found in 5 of the 9 newborns. Four severely damaged newborns died within 12–72 h after delivery. Hemorrhagic necrosis occurred in the cerebral cortex, basal ganglia, and thalamus of both hemispheres, and these changes were associated with pronounced brain swelling and herniation of the cerebellar tonsils. These findings indicate that even a single maternally acute exposure to perinatal CO at levels which were tolerated by dams can cause death and neurological damage in newborns.

5. Carbonyl fluoride (CF)

CF is a colorless gas with a pungent and very irritating odor, and categorized as a toxic gas and vapor [47]. CF is extremely hygroscopic and rapidly hydrolyzed in contact with water, and this hydrolysis occurs in humid air [48].

A TLV-TWA of 2 ppm and TLV-STEL of 5 ppm [46] are recommended for CF (Table 1).

CF is highly corrosive to the skin and mucous membranes [47]. Inhalation of CF is the major route of entry. When inhaled into the respiratory tract covered with mucous fluid, CF is hydrolyzed to yield CO_2 and two molecules of HF [48,49]. Toxic effects of inhaled CF indeed resembled the toxic effects of HF, and the toxic action of CF was concluded to be based on the toxic action of HF [49]. The toxicity of CF has been shown to be about that of HF. No reports are available on the reproductive and developmental toxicity of CF.

6. Hydrogen fluoride (HF)

HF is a colorless gas at temperatures above its boiling point and a fuming liquid at temperatures below its boiling point, and has a strong irritating odor that is discernible at a concentration of about 0.04 ppm [50]. Natural sources of HF include volcanoes, the weathering of minerals and marine aerosols. Industrial sources include the production of HF itself and by-products of the production of phosphate fertilizer, aluminum and steel, and ceramics.

A TLV-TWA of 0.5 ppm, TLV-STEL of 2 ppm [50], IDLH of 30 ppm [51], measured as fluoride (F), and OEL of 3 ppm [23] are recommended for HF (Table 1).

Table 5
Testicular and sperm toxicity studies of sodium fluoride in male experimental animals.

Animals	Treatment			Findings	References
	Rout	Frequency/length	Dose		
Swiss mice	Gavage	30 days	10 mg/kg bw/day	Alteration in male reproductive organs (transient)	[53]
Swiss mice	Oral	30 days	10 mg/kg bw/day	↓Epididymal sperm motility and count (transient) ↓Fertility rate (transient)	[54]
Rat	Oral	30 days	5 mg/kg bw/day	↓Epididymal sperm count ↓fertility rate ↓Succinate dehydrogenase (SDH) in testes	[55]
			10 mg/kg bw/day	↓Epididymal sperm motility ↓ATPase in epididymis	
SD rat	Intratesticular injection	Single	50 μL of 250 ppm solution	No adverse effects on spermatogenesis	[56]
Wistar rat	Gavage	29 days	20 mg/kg bw/day	↓Testes, prostates and seminal vesicles wt ↓Epididymal sperm count ↓serum testosterone ↓Number of mature luminal spermatozoa ↓3β- and 17β-hydroxysteroid dehydrogenase (HSD) ↑Conjugated dienes in testes, epididymides and sperm pellet	[57]
Wistar rat	Drinking water	75 days	4.5 ppm	↓Epididymal sperm count ↓sperm viability	[58]
			(0.7 mg/kg bw/day)	↓Sperm motility ↓hypoosmotic sperm coiling ↓3β- and 17β-HSD ↑abnormal sperm	
			9.0 ppm (1.3 mg/kg bw/day)	↓Body wt	
Rabbit	Oral	30 days	20 mg/kg bw/day	↓Epididymal sperm count ↓sperm motility ↓ATPase, succinate SDH, acid phosphatase, total protein and N^+ and K^+ in spermatozoa	[59]

Fluoride (F) can be found in all tissues in the body after oral, inhaled or dermal exposure to HF, and sequestration takes place in bone tissue in which about half of the absorbed F is deposited [52]. The most important route of exposure for plants is uptake from the atmosphere, and consumption of F-containing plants results in elevation of F levels in humans and animals. Total daily F intake of human adults in a worst case scenario is estimated to be 5990 μg/day including 5640 μg/day from food and drinking water, 50 μg/day from air and 300 μg/day from toothpaste, and animal feed for routine toxicity studies contains 20 mg/kg, approximately 1 mg/kg bw/day [52].

There is no information available on the reproductive and developmental toxicity of HF. Data on sodium fluoride (NaF) were used to obtain insight into the reproductive and developmental toxicity of HF because NaF is similar in kinetics and dynamics to HF [52].

Testicular and sperm toxicity studies of NaF are presented in Table 5. Male Swiss mice were given NaF by gavage at 10 or 20 mg/kg bw/day for 30 days [53]. NaF caused a severe disorganization and denudation of germinal epithelial cells of the seminiferous tubules with an absence of sperm in the lumen. Epitherial cell nuclear pyknosis and an absence of luminal sperm in the caput epididymis, and a reduction in epitherial cell height, nuclear pyknosis, the denudation of cells and an absence of sperm in the cauda epididymis were observed following the administration of NaF. Nuclear pyknosis, clumped stereocillia and cell debris were found in the vas deferens of NaF-treated mice. A marked recovery in the histoarchitecture of these organs was noted after a 2-month withdrawal period. These results indicate that effects of NaF are transient and reversible and NaF does not cause any permanent structural alterations in the reproductive organs in mice. Male Swiss mice received oral NaF at 10 or 20 mg/kg bw/day for 30 days [54]. Although decreases in cauda epididymal sperm motility and counts and in the fertility rate were observed after the 30-day administration, withdrawal of NaF-treatment for 2 months resulted in a recovery of these parameters. In rats, NaF was orally given at 5 or 10 mg/kg bw/day to males for 30 days [55]. Decreases in the epididymal sperm count and fertility rate at 5 and 10 mg/kg bw/day and in epididymal sperm motility at 10 mg/kg bw/day were found. Inhibition of succinate dehydrogenese (SDH) activity in the testes of NaF-treated males indicated a hampered testicular oxidative metabolism in NaF-treated rats which could substantially affect the spermatogenesis. The left testis of each SD rat was injected with 50 μL of a 50–250 ppm NaF solution, and histopathological examinations of the testes were performed [56]. Although the authors concluded that direct exposure to NaF had no adverse effects on spermatogenesis at levels 200 times greater than those under normal conditions, the findings of this study cannot be interpreted as indicating no testicular toxicity of oral NaF. Male Wistar rats received NaF by gavage at 20 mg/kg bw/day for 29 days [57]. NaF caused decreases in the relative weights of the testis, prostate and seminal vesicle, activities of 3β- and 17β-hydroxysteroid dehydrogenase (HSD), plasma testosterone levels, epididymal sperm count and number of mature luminal spermatozoa, but not changes in the body weight gain. These findings were associated with the induction of oxidative stress as indicated by increased levels of conjugated dienes in the testes, epididymides and sperm pellet. NaF in drinking water caused decreases in the epididymal sperm count, sperm viability, sperm motility, hypoosmotic sperm coiling percentage and 3β- and 17β-HSD activities and an increase in the percentage of abnormal sperm at 4.5 ppm (0.7 mg/kg bw/day) and decreased body weight at 9.0 ppm (1.3 mg/kg bw/day) in male Wistar rats given NaF for 75 days [58]. In rabbits, epididymal sperm counts and motility and the fertility rate were decreased in males given oral NaF at 20 and 40 mg/kg/day for 30 days [59]. A complete loss of fertility was found at 40 mg/kg bw/day. ATPase, SDH, acid phosphatase, total protein and N^+ and K^+ levels in the spermatozoa were reduced at 20 and 40 mg/kg bw/day. None of these parameters returned to normal values after a one-month recovery period. The findings of the above studies suggest that the rat is the species most susceptible to the testicular and sperm toxicity of fluoride and

Table 6
Reproductive and developmental toxicity studies of sodium fluoride in experimental animals.

Animals	Treatment			Findings	References
	Rout	Frequency/length	Dose		
SD rat	Drinking water	2 generations	250 ppm	No effect on male reproductive organ wt or sperm of P or F_1	[60]
			(24 mg/kg bw/day)	No effect on morphometry in testes of F_1	[61]
CD rat	Drinking water	2 generations	250 ppm	No effect on reproduction or development of P or F_1	[62]
			(24–28 mg/kg bw/day)		
CD rat	Drinking water	2 generations	250 ppm (28 mg/kg bw/day)	↑Skeletal retardation in F_2 fetuses	[63]
Wistar rat	Drinking water	3 generations	10 mg/L (10 ppm) (2 mg/kg bw/day)	↓Lung relative wt of males	[64]
			50 mg/L (50 ppm) (10 mg/kg bw/day)	↓Body wt of F_2 males Degenerative change in lungs of F_2 males	
			100 mg/L (100 ppm) (19 mg/kg bw/day)	No effect on reproduction or offspring survival	
Wistar rat	Drinking water	3 generations	50 mg/L (50 ppm) (10 mg/kg bw/day)	Histopathological degenerative change in myocardial tissues of F_2 males Biochemical oxidative change in myocardial tissues of F_2 males	[65]
Wistar rat	Drinking water	3 generations	30 mg/L (30 ppm) (6 mg/kg bw/day)	Destruction of kidney tissues in F_1 and F_2 males	[66]
CD rat	Drinking water	GDs 0–20	175 ppm (24.7 mg/kg bw/day)	↓Maternal water consumption	[67]
			250 ppm (25.1 mg/kg bw/day)	↓Maternal food consumption and body wt gain ↑Fetal skeletal variation	
SD rat	Drinking water	GDs 6–15	300 ppm (27.1 mg/kg bw/day)	↓Maternal water consumption No effect on fetal development	[68]
Wistar rat	Oral	GDs 6–21	40 mg/kg bw/day	↑Serum Na, K, and P in P and F_1 (transient) ↓Serum Ca in P and F_1	[69]
NZW rabbit	Drinking water	GDs 6–19	400 ppm (29.2 mg/kg bw/day)	↓Maternal water consumption No effect on fetal development	[68]

adverse effects on the testis and sperm are due to the induction of oxidative stress and decreased steroidogenesis.

Reproductive and developmental toxicity studies of NaF are shown in Table 6. Multiple-generation studies are available. SD rats were given drinking water containing NaF at 25, 100, 175 or 250 ppm for 14 weeks (10 weeks before mating, during 3 weeks of mating and 1 week after mating). Males and females within a treatment group were mated. Pregnant females (P) continued to be given NaF throughout gestation and lactation [60]. The weanlings (F_1) remained in the same treatment groups as their parents and were given NaF for 14 weeks. Even at 250 ppm (24 mg/kg bw/day), no adverse effects were observed in weights of organs including the reproductive organs, sperm parameters, serum LH, FSH and testosterone levels or the histopathology of the testes in P or F_1 males [60] or in the quantitative morphometry of the testes in F_1 males [61]. NaF was given to CD rats with the same regimen and procedure as above [60], and some pregnant P and F_1 females were sacrificed to examine the development of F_1 and F_2 fetuses, respectively, on GD 20 [62,63]. Although no adverse effects on reproduction or development were noted even at the highest dose of 250 ppm (24–28 mg/kg bw/day) [62], decreased ossification of the hyoid bone of F_2 fetuses was found at 250 ppm (28 mg/kg bw/day) [63]. Drinking water containing NaF at 10, 50 or 100 mg/L (2, 10 or 19 mg/kg bw/day) was given to Wistar rats throughout gestation and lactation in the P generation through to 6 months after weaning in the F_2 generation, and F_2 males were examined [64]. Decreases in the relative weight of the lung at 10 mg/L and higher, and body weight at 50 mg/L and higher were observed. In the lung at 50 and 100 mg/L, histopathologically degenerative changes associated with biochemical change suggested oxidative damage. However, NaF had no effects on the reproduction or survival of offspring even at 100 mg/L [64]. Histopathologically degenerative changes were associated with biochemical changes suggesting oxidative damage in the myocardium of F_2 males at 50 and 100 mg/L [65]. Wistar rats received NaF in drinking water at 30 mg/L (6 mg/kg bw/day) throughout gestation and lactation in the P generation through to 4 months after weaning in the F_2 generation [66]. Chronic NaF produced marked destruction of kidney tissue of F_1 and F_2 males by causing lipid peroxidation.

The morphological development of fetuses of dams given NaF during gestation was studied. CD rats received NaF in drinking water at 10, 25, 100, 175 or 250 ppm (1.4, 3.9, 15.6, 24.7 or 25.1 mg/kg bw/day) throughout gestation [67]. Water consumption at 175 ppm and water and food consumption and body weight gain at 250 ppm were decreased in dams. No effect of NaF was found on fetal weight or length. Although the number of fetuses with three or more skeletal variations was increased at 250 ppm, no teratogenicity of NaF was noted. NaF was given in drinking water to SD rats on GDs 6–15 at 50, 150 or 300 ppm (6.6, 18.3 or 27.1 mg/kg bw/day) and to New Zealand White rabbits on GDs 6–19 at 100, 200 or 400 ppm (10.3, 18.1 or 29.2 mg/kg bw/day) [68]. At the highest dose, decreased water consumption due to poor palatability was observed in rats and rabbits. No reproductive or developmental effects were noted in either species. Wistar rats received NaF orally at 40 mg/kg bw/day from GD 6 throughout lactation or gestation only which was followed by a withdrawal of NaF-treatment, and blood samples were collected from P and F_1 rats on day 21 of lactation [69]. Decreased levels of serum sodium, potassium and phosphorus and increased levels of serum calcium were observed in P and F_1 rats given NaF during gestation only, and alterations in cationic concentrations, except for calcium, recovered after the withdrawal of NaF-treatment. These results suggest that hypocalcemia could be responsible for skeletal alterations in developing fetuses. The results of developmental toxicity studies, in which NaF

was given during gestation including organogenesis, revealed that NaF affected development, but was not teratogenic.

7. Formic acid (FA)

FA is a colorless gas with a pungent and penetrating odor [70]. Exposure to FA can occur through inhalation, ingestion, and contact via the eyes or skin. The primary physiological characteristic of FA is an irritating action on the mucous membrane, eyes and skin [71].

A TLV-TWA of 5 ppm, TLV-STEL of 10 ppm [71], IDLH of 30 ppm [70] and OEL of 5 ppm [23] are recommended for FA (Table 1).

FA was dysmorphogenic and developmentally toxic to rat and mouse embryos in a whole embryo culture system [72]. No report is available on the reproductive and developmental toxicity of FA in vivo.

8. Discussion and conclusions

In this paper, we summarized the reproductive and developmental toxicity of degradation products of refrigerants, including TFA, CO_2, CO, CF, HF and FA, in experimental animals. The risk assessment of chemicals is difficult because there are many variables in the manifestation of reproductive and developmental toxicity. However, confirmation of adverse effects on reproduction and development in animals exposed by the anticipated route of human exposure would be important for risk assessment in humans.

Excessive exposure to CO_2 in humans is well reported, but there is a lack of information on reproductive and developmental toxicity [73]. In experimental animals, excessive CO_2 in the atmosphere is testicular and reproductive toxic, embryolethal, developmentally neurotoxic and teratogenic. The data for these animal studies suggest the adverse effects of CO_2 on reproduction and development to be due to a secondary effect such as acidosis, increased blood flow or increased oxygen tension.

Although human studies on CO are limited, developmental toxicity has mainly been confined to the central nervous system [74]. The inhalation of CO has not proved to be consistently teratogenic in animals, and only very early studies in rats and guinea pigs reported congenital malformations: more recent studies have not [74,75]. Studies in several species of animals showed that maternal exposure to CO cause prenatal and postnatal lethality and growth retardation, increased incidence of skeletal variations, cardiomegaly, blood biochemical changes, immunological changes, postnatal behavioral changes, and neurological impairment in offspring of exposed dams. Some of these changes in fetuses and pups were detected at levels which did not cause maternal toxic effects. Even at levels as low as 60–65 ppm, maternal exposure to CO can cause cardiomegaly and delayed reflex ontogeny in rat offspring. COHb levels in fetuses and pups increase to above the levels in the maternal circulation after maternal CO exposure. A further decrease in oxygen tension due to the presence of COHb could have potentially serious effects in fetuses with a lower oxygen tension, and increased COHb levels could have hypoxic effects in newborn pups with a high rate of oxygen consumption and lower oxygen transport capacity for hemoglobin [31]. These phenomena indicate that fetuses and pups are susceptible to CO exposure. Further studies are therefore required to evaluate the adverse effects of chronic exposure to low and near ambient levels of CO on the development of fetuses and newborn pups.

Little information is available on the toxicity of CF and HF. However, the results of toxicological studies on NaF have been used to obtain insight into the toxicity of CF and HF, because CF is rapidly hydrolyzed in contact with water and yields CO_2 and HF [48,49] and NaF is similar in kinetics and dynamics to HF. Developmental toxicity studies, in which NaF was administered to rats and rabbits during gestation including organogenesis, revealed an increased incidence of fetal skeletal variations, but not fetal malformations. Rat multiple-generation toxicity studies revealed that NaF retarded ossification in F_2 fetuses, caused degenerative changes in the lung and kidney of F_2 male offspring, but had no adverse effects on parameters for reproductive toxicity including testicular or sperm toxicity. There is a discrepancy in testicular and sperm toxicity between regimens of NaF. Testicular and sperm toxicity was noted when NaF was directly given to young or adult rats. The efficiency of sperm production and epididymal sperm reserves of humans are considerably lower than those of experimental animal models [76]. It is also noted that human males have relatively low fertility and thus may be at greater risk from reproductive toxicants than experimental animals [77]. Furthermore, male rodents produce sperm in numbers that greatly exceed the minimum requirement for fertility while sperm production in human males appears to be closer to the infertility threshold, therefore a less severe reduction in sperm counts may affect male fertility [78]. These considerations suggest that definitive animal studies of chemicals suspected of having testicular and sperm toxicity are needed to assess the risk to reproduction in human males. Further histopathological studies of reproductive organs given NaF could help us to understand the reproductive toxicity of NaF, because histopathology is acknowledged as the most sensitive endpoint for detecting testicular toxicity [79].

There is a lack of information on the toxicity of TFA and FA.

Evidence from human studies is preferred for risk assessment as long as it is obtained humanely. It is sometimes claimed that the use of animal data for estimating human risk does not provide strong scientific support. However, a continuance of studies in experimental animals is required for risk assessment of chemicals because it is difficult to find alternative methods to test the direct toxic effects of chemicals.

Conflict of interest

None.

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Prenatal developmental toxicity of gavage or feeding doses of 2-*sec*-butyl-4,6-dinitrophenol in rats

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ABSTRACT

This study evaluated the prenatal developmental toxicity of the pesticide 2-*sec*-butyl-4,6-dinitrophenol (dinoseb). Pregnant rats were given dinoseb by gavage at 0, 8.0 or 10 mg/kg bw/day on days 6–15 of gestation, or in the diet at 0, 120 or 200 ppm (0, 6.52 or 8.50 mg/kg bw/day) on days 6–16 of gestation, and litters were evaluated on day 20 of gestation. Maternal toxicity was observed as evidenced by significantly decreased body weight gain and reduced food consumption during the administration period in all the dinoseb-treated groups, and two dams died at 10 mg/kg bw/day. Significantly lower fetal weights and delayed skeletal ossification was observed in the dinoseb-treated groups except for the group fed dinoseb at 120 ppm. The teratogenic potential of the gavage dose of dinoseb was confirmed as evidenced by increased incidences of fetuses with external and skeletal malformations at 10 mg/kg bw/day. The incidence of fetuses with microphthalmia was significantly increased at this dose. On the other hand, feeding doses of dinoseb up to 200 ppm did not induce teratogenicity in this study. These data indicate that dinoseb is teratogenic at maternally toxic doses, but the exposure range of dinoseb at which malformations occur seems to be narrow.

1. Introduction

Dinoseb, 2-*sec*-butyl-4,6-dinitrophenol (CAS No. 88-85-7) was approved for sale in the US in 1948 as a nitrophenolic herbicide in soybeans, vegetables, fruits, nuts, citrus and other field crops for the selective control of grass and broadleaf weeds [1,2]. Dinoseb is also used as an insecticide for grapes and as a seed crop-drying agent [2]. Dinoseb is one of the chemicals permitted on the market on the basis of safety tests conducted by Industrial Bio-Test Laboratory, a concern later found to have submitted many flawed and even fraudulent reports on its procedures and results [3]. Subsequently, several studies showed that dinoseb has the potential to produce developmental toxicity including teratogenicity in rats, mice and rabbits [4–7].

Dinoseb as a pesticide was banned in the US in 1986 and the EU in 1991 owing to the potential risk of adverse health effects in humans [2,8], but dinoseb and its salts are still widely used as other agricultural products [9]. Dinoseb is a high volume chemical with production or importation exceeding 1000 tons/year in Organisation for Economic Co-operation and Development (OECD) member countries [10]. Dinoseb as a pesticide is also banned in Japan but its import is permitted [9], and the volumes of dinoseb imported into Japan were estimated to be 827 tons in fiscal year 2007 and 615 tons in fiscal year 2008 [11].

Exposure to dinoseb may occur by direct contact, ingestion or inhalation by users and producers. Indirect exposure to dinoseb via the environment is also anticipated. The microbial breakdown of dinoseb has been demonstrated in soils, but dinoseb persists for about 2–4 weeks after application [12]. A soil persistence of 24–42 months was also observed in potato fields in Canada [13]. It has been reported that dinoseb was detected in water supplies in Canada and the US, and dinoseb residues were found in a cotton meal sample [12].

In previous review papers, we showed that dinoseb possesses testicular toxicity [14] and developmental toxicity [15] in experimental animals. We reported the results of a combined repeated dose toxicity study with a reproduction/developmental toxicity screening test, in which Crj:CD(SD)IGS rats were administered dinoseb by gavage at 0, 0.78, 2.33 or 7.0 mg/kg bw/day. At 7.0 mg/kg bw/day, eight females died and two animals were moribund during late pregnancy, and a significant decrease in body weight gain was found in both sexes. The numbers of dams that delivered their pups and dams with live pups at delivery were significantly reduced at this dose. Because only two females in the highest dose group delivered their pups, the developmental toxicity of dinoseb was not fully assessed in this study [16], but gross internal and external examinations revealed no significant differences in the incidence of pups with malformations. In a previous review

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[15], we concluded that teratogenic susceptibility to dinoseb was greater in rabbits than in rats and mice. Several studies failed to demonstrate the teratogenicity of dinoseb in rats [16–18], but we consider that the teratogenic potential of dinoseb in rats is unclear for various reasons. The feeding dose of dinoseb to rats on days 5–14 of gestation increased the incidence of fetuses with microphthalmia at 200 ppm (15 mg/kg bw/day), but this was not observed by gavage dosing at 15 mg/kg bw/day [4]. The incidence of fetuses with microphthalmia also increased when dinoseb was given in a certain composition of diet (protein 21%, fat 4.8%, fiber 4.2%, ash 8.5% and N-free extractives 61.5%) at 200 ppm or by gavage with the same diet at 15 mg/kg bw/day on days 5–13 of gestation, but this effect was not observed when a different diet (protein 21%, fat 3.5%, fiber 6.5%, ash 7.5% and N-free extractives 61.5%) was fed to pregnant rats [19]. As described above, adequate experimental conditions for the production of fetal malformations by the administration of dinoseb to pregnant rats remain unknown. Therefore, the present study was conducted to clarify the experimental conditions that produce fetal malformations when dinoseb is given to pregnant rats.

2. Materials and methods

2.1. Animals

This study was performed in 2008 at the Safety Research Institute for Chemical Compounds Co., Ltd. (Sapporo, Japan). The study was conducted in accordance with "Act on Welfare and Management of Animals" [Act No. 105, October 1, 1973, revised December 22, 1999, Revised Act No. 221; revised June 22, 2005, Revised Act No. 68], "Standards Relating to the Care, Management and Refinement of Laboratory Animals" [Notification No. 88 of the Ministry of the Environment, Japan, April 28, 2006] and "Basic Policies for the Conduct of Animal Experiments in Research Institutions under the Jurisdiction of the Ministry of Health, Labour and Welfare" [Notification No. 0601005 of the Health Sciences Division, Ministry of Health, Labour and Welfare, Japan, June 1, 2006].

Male and female SPF Crl:CD (SD) rats were used throughout this study. This strain was chosen because it is most commonly used in toxicity studies, and historical control data are available. Rats at 8 weeks of age were purchased from Atsugi Breeding Center, Charles River Laboratories Japan, Inc. (Yokohama, Japan) and were quarantined and acclimated to the laboratory for 3 weeks prior to the start of the experiment. Male and female rats found to be in good health were selected for use. The animals were reared on a sterilized basal diet (CRF-1; protein 22%, fat 5.7%, fiber 2.9%, ash 6.3% and N-free extractives 55.3%; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water *ad libitum*. After the quarantine and acclimation, male and female rats were housed individually except during the mating period. Daily vaginal lavage samples of each female were evaluated for estrous cyclicity. Females showing proestrous vaginal smears were paired overnight with males on a 1:1 basis. The females were examined next morning for vaginal plugs and sperm in vaginal smears. The day on which the presence of sperm in the vaginal smear and/or a vaginal plug was detected was designated day 0 of gestation. The mated females were separated into three groups to equalize the female body weights in the gavage dose groups or the feeding dose groups. The animals were maintained in an air-conditioned room at a room temperature of 22 ± 3 °C, a relative humidity of 50 ± 20%, a 12-hour light/dark cycle and 10–15 air changes per hour.

2.2. Chemicals and dosing

Dinoseb was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The dinoseb (Lot No. 010608LB-AC) used in this study was 100% pure, and was stored under refrigeration prior to use. The purity and stability of the chemical were verified by analysis before the study. Dose levels were determined on the basis of the results of studies by Giavini et al. [4,19]. At these doses, maternal and/or developmental toxicity was/were expected to be observed in the dinoseb-treated groups. For the gavage dose groups, 12 females per group were given dinoseb once daily by gastric intubation at 0 (control), 8.0 and 10 mg/kg bw from day 6 to day 15 of gestation. The dinoseb was suspended in corn oil, and the control rats were given only corn oil. The volume of each dose was adjusted to 5 ml/kg body weight on the basis of the latest body weight. The dosing suspensions were prepared once per 7 days, and were stored in the dark and cold conditions before use. For the feeding dose groups, 12 females per group were given dinoseb in the diet from day 6 to day 16 of gestation at 0 (control), 120 and 200 ppm, and were thus expected to consume similar amounts of dinoseb to those in the gavage groups. The control rats were given the basal diet. The diet for the dose groups was prepared more than once every 4 days and was stored at room temperature before use.

2.3. Observations

All female rats were observed for clinical signs of toxicity once a day before and after the administration period, twice a day during the administration period and once on the day of sacrifice. Body weight was recorded once a day during the administration period and on days 0, 18 and 20 of gestation, and body weight gain was calculated. Food consumption was recorded on days 0, 6, 9, 12, 16, 18 and 20 of pregnancy. Rats that died during the administration period were autopsied and grossly examined. The pregnant rats were killed by exsanguination under ether anesthesia on day 20 of gestation. The organs and tissues were grossly examined. The ovary and uterus were removed from the maternal body, and gravid uterine weight was recorded. The numbers of corpora lutea, implantation sites, live and dead fetuses and resorptions were recorded. The placenta was removed and weighed. The live fetuses were removed from the uterus, sexed, weighed and inspected for external malformations and malformations within the oral cavity. The live fetuses were put down using an intraperitoneal injection of a sodium pentobarbital solution, and the eyes of the fetuses were examined after the removal of the skin of the head. Then, approximately one-half of the live fetuses in each litter were fixed in Bouin's solution for the examination of internal anomalies. Their heads were subjected to free-hand razor-blade sectioning [20], and the thoracic areas were subjected to microdissection [21]. The remaining live fetuses in each litter were fixed in 70% ethanol, stained with Alizarin red S and alician blue, and examined for skeletal anomalies.

2.4. Data analysis

Maternal body weight gain, gravid uterine weight, food consumption, number of corpora lutea, number of implantations, number of live fetuses, number of dead or resorbed embryos/fetuses, fetal weight, placental weight and degree of ossification were analyzed for statistical significance as follows. Bartlett's test of homogeneity of variance was used to determine if the groups had equivalent variances at the 5% level of significance. If the variances were equivalent, the groups were compared by one-way analysis of variance. If significant differences were found, Dunnett's multiple comparison test was performed. If the group variances were not equivalent, the Kruskal–Wallis test was used to assess the overall effects. Whenever significant differences were noted, pair-wise comparisons were made using the Mann–Whitney *U*-test. Fetal weight, placental weight and degree of ossification were analyzed using the litter as a unit. Implantation index, viability index of fetuses, total incidence of dead or resorbed embryos/fetuses, incidence of fetuses with malformations or variations and sex ratio of live fetuses were analyzed by Wilcoxon's rank sum test using the litter as a unit. The 0.05 level of probability was used as the criterion for significance.

3. Results

Table 1 shows the maternal findings in rats given dinoseb by gavage or in the diet. At 10 mg/kg bw/day, death occurred on days 10 and 13 of gestation in one female each. No changes in clinical findings were observed in the feeding dose and the other gavage dose groups. Maternal body weight gain was significantly decreased on days 6–16 and 0–20 of gestation in all the dinoseb-treated groups and significantly increased on days 16–20 of gestation at 200 ppm. Food consumption was significantly decreased in the gavage dose groups on days 6–9 and 9–12 of gestation at both 8.0 and 10 mg/kg bw/day. After the administration period, food consumption was increased at 8.0 and 10 mg/kg bw/day, and a significant increase was observed on days 16–18 of gestation at 8.0 mg/kg bw/day. Similarly, food consumption was significantly decreased during the administration period in the feeding dose groups at 120 and 200 ppm, and it was significantly increased at 200 ppm after the administration period. The average intakes of dinoseb at 120 and 200 ppm were 6.52 and 8.50 mg/kg bw/day, respectively. At autopsy, dilatation of renal pelvis was observed in only one rat at 8.0 mg/kg bw/day, which was suggested to be spontaneous occurrence. Two animals that died during the administration period at 10 mg/kg bw/day showed abnormal findings such as discoloration of the lung and spleen, atrophy of the thymus, thickening limiting ridge of the stomach and/or dark red patch in the glandular stomach. No changes were observed in the feeding dose groups at autopsy (data not shown).

Table 2 presents the reproductive findings in rats given dinoseb by gavage or in the diet. Body weights of live fetuses were decreased in the dinoseb-treated groups, and significantly decreased body weights were noted in male fetuses at 10 mg/kg bw/day, in

Table 1
Maternal findings in rats given dinoseb by gavage or in the diet.

Dose	Gavage dose			Feeding dose		
	Control	8.0 mg/kg	10 mg/kg	Control	120 ppm	200 ppm
No. of pregnant rats	12	12	12	12	12	12
Initial body weight	263.3 ± 10.4[a]	263.7 ± 10.0	262.8 ± 11.0	299.1 ± 21.8	298.9 ± 22.9	298.9 ± 25.9
No. of females showing clinical signs of toxicity						
Death	0	0	2	0	0	0
Body weight gain during pregnancy (g)						
Days 0–6	42.3 ± 7.9	36.8 ± 5.7	39.6 ± 5.4	27.1 ± 5.8	26.3 ± 7.4	24.2 ± 6.4
Days 6–16	59.3 ± 9.5	31.3 ± 7.4**	25.6 ± 8.2** (10)	48.7 ± 12.9	25.3 ± 5.2**	−11.4 ± 5.8**
Days 16–20	67.1 ± 8.4	70.8 ± 9.8	68.8 ± 9.9 (10)	64.1 ± 9.9	64.3 ± 9.8	81.4 ± 15.1**
Days 0–20	168.8 ± 18.4	138.9 ± 12.4**	133.3 ± 14.7** (10)	139.8 ± 20.1	115.9 ± 14.8**	94.2 ± 19.9**
Food consumption during pregnancy (g/day)						
Days 0–6	23.4 ± 1.8	22.9 ± 1.6	23.3 ± 1.4	21.5 ± 2.1	22.2 ± 2.5	20.4 ± 1.9
Days 6–9	21.0 ± 1.9	17.1 ± 1.4**	16.2 ± 2.4**	21.1 ± 2.3	16.8 ± 0.9**	12.0 ± 1.1**
Days 9–12	22.3 ± 2.2	19.7 ± 1.7*	19.5 ± 2.8** (11)	21.8 ± 4.2	17.2 ± 1.4**	11.7 ± 1.5**
Days 12–16	21.5 ± 2.1	20.5 ± 1.1	22.1 ± 1.9 (10)	22.4 ± 2.4	20.6 ± 3.2	15.6 ± 2.0**
Days 16–18	25.5 ± 2.2	28.2 ± 2.1**	27.6 ± 1.9 (10)	24.0 ± 2.5	25.2 ± 2.6	28.2 ± 2.9**
Days 18–20	26.3 ± 1.5	27.9 ± 2.6	28.0 ± 1.6 (10)	23.1 ± 2.6	24.2 ± 2.6	27.3 ± 2.8**

Values in parentheses are the number of animals examined.
[a] Values are given as the mean ± SD.
* Significantly different from the control ($p < 0.05$).
** Significantly different from the control ($p < 0.01$).

female fetuses at 8.0 and 10 mg/kg bw/day and in both sexes at 200 ppm. Weight of the placenta was significantly decreased at 10 mg/kg bw/day, but it was not affected by the feeding dose of dinoseb. Gravid uterine weight was decreased dose-dependently. No effects were observed in other reproductive parameters.

The summarized results of external and internal examinations of fetuses are shown in Table 3. External malformations were found in 1 out of the 171 fetuses (1 out of 12 litters) at 8.0 mg/kg and 18 out of the 147 fetuses (4 out of the 10 litters) at 10 mg/kg bw/day, and the incidence of fetuses with external malformations was significantly increased at 10 mg/kg bw/day. Among the fetuses at 10 mg/kg bw/day, there were 1 each with cleft palate or filamentous tail, 2 each with runt, anotia, brachymelia or ectrodactyly and 17 fetuses with microphthalmia. The incidence of fetuses with microphthalmia was significantly increased at this dose. No significant differences were found upon external examinations of the feeding dose groups. Runt was observed in one fetus at 8 mg/kg bw/day and each one fetus in two different litters at 10 mg/kg bw/day. In the internal examinations, no significant differences were observed in the gavage and feeding dose groups.

The summarized results of skeletal examinations of the fetuses are presented in Table 4. There were no significant differences between the dinoseb-treated and control groups in the incidence

Table 2
Reproductive findings in rats given dinoseb by gavage or in the diet.

Dose	Gavage dose			Feeding dose		
	Control	8.0 mg/kg	10 mg/kg	Control	120 ppm	200 ppm
No. of litters	12	12	10	12	12	12
No. of corpora lutea per litter	16.3 ± 2.3[a]	16.0 ± 2.2	15.9 ± 1.7	15.5 ± 1.6	15.4 ± 1.0	13.9 ± 2.9
No. of implantations per litter	14.9 ± 3.4	14.8 ± 2.5	15.2 ± 2.2	15.2 ± 1.9	14.4 ± 1.1	13.6 ± 3.0
Implantation index (%)[b]	90.5 ± 14.8	92.6 ± 12.5	95.4 ± 6.7	97.8 ± 4.4	93.6 ± 5.8	97.5 ± 3.7
Dead or resorbed embryos and fetuses						
Early stage[c]	0.4 ± 0.5	0.5 ± 0.5	0.4 ± 0.5	0.8 ± 0.7	0.7 ± 0.8	0.8 ± 1.3
Late stage[d]	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Total incidence (%)[e]	2.7 ± 3.4	3.1 ± 3.2	3.4 ± 3.7	5.4 ± 4.4	4.7 ± 5.5	6.6 ± 9.0
No. of live fetuses	14.5 ± 3.4	14.3 ± 2.1	14.7 ± 2.3	14.3 ± 1.7	13.8 ± 1.4	12.8 ± 3.2
Viability index of fetuses (%)[f]	97.3 ± 3.4	96.9 ± 3.2	96.6 ± 3.7	94.6 ± 4.4	95.4 ± 5.5	93.4 ± 9.0
Sex ratio of live fetuses[g]	0.472 ± 0.152	0.472 ± 0.136	0.447 ± 0.163	0.503 ± 0.133	0.506 ± 0.141	0.427 ± 0.152
Body weight of live fetuses (g)						
Male	4.043 ± 0.283	3.792 ± 0.285	3.425 ± 0.279**	4.033 ± 0.293	3.858 ± 0.281	3.620 ± 0.217**
Female	3.873 ± 0.228	3.587 ± 0.221*	3.240 ± 0.315**	3.780 ± 0.288	3.641 ± 0.253	3.399 ± 0.261**
Gravid uterine weight (g)	84.3 ± 19.1	78.9 ± 11.2	74.7 ± 11.5	84.1 ± 12.7	77.5 ± 8.5	70.1 ± 18.4*
Placental weight (g)	0.483 ± 0.047	0.467 ± 0.030	0.435 ± 0.046*	0.502 ± 0.045	0.477 ± 0.037	0.518 ± 0.096

[a] Values are given as the mean ± SD.
[b] (Number of implantations/number of corpora lutea) × 100.
[c] Includes implantation sites and placental remnants.
[d] Includes macerated fetuses and dead term fetuses.
[e] (Number of dead or resorbed embryos and fetuses/number of implantations) × 100.
[f] (Number of live fetuses/number of implantations) × 100.
[g] Number of live male fetuses/number of live fetuses.
* Significantly different from the control ($p < 0.05$).
** Significantly different from the control ($p < 0.01$).