Industrial Health 2002, **40**, 295–311

Review Article

Apportionment in Asbestos-Related Disease for Purposes of Compensation

Tee L. GUIDOTTI

Department of Public Health Sciences, University of Alberta, Faculty of Medicine & Dentistry, Edmonton, Alberta, Canada

Present: Division of Occupational and Environmental Medicine, The George Washington University Medical Center, 2300 K Street, NW, Suite 201, Washington DC 20036, U.S.A.

Received October 13, 2000, revised June 1, 2001, and accepted July 26, 2002

Abstract: Workers' compensation systems attempt to evaluate claims for occupational disease on an individual basis using the best guidelines available to them. This may be difficult when there is more than one risk factor associated with the outcome, such as asbestos and cigarette smoking, and the occupational exposures is not clearly responsible for the disease. Apportionment is an approach that involves an assessment of the relative contribution of work-related exposures to the risk of the disease or to the final impairment that arises for the disease. This article discusses the concept of apportionment and applies it to asbestos-associated disease. Lung cancer is not subject to a simple tradeoff between asbestos exposure and smoking because of the powerful biological interaction between the two exposures. Among nonsmokers, lung cancer is sufficiently rare that an association with asbestos can be assumed if exposure has occurred. Available data suggest that asbestos exposure almost invariably contributes to risk among smokers to the extent that a relationship to work can be presumed. Thus, comparisons of magnitude of risk between smokers and nonsmokers are irrelevant for this purpose. Indicators of sufficient exposure to cause lung cancer are useful for purposes of establishing eligibility and screening claims. These may include a chest film classified by the ILO system as 1/0 or greater (although 0/1 does not rule out an association) or a history of exposure roughly equal to or greater than 40 fibres/cm^3·y. (In Germany, 25 fibres/cm^3·y is used.) The mere presence of pleural plaques is not sufficient. Mesothelioma is almost always associated with asbestos exposure and the association should be considered presumed until proven otherwise in the individual case. These are situations in which only risk of a disease is apportioned because the impairment would be the same given the disease whatever the cause. Asbestosis, if the diagnosis is correct, is by definition an occupational disease unless there is some source of massive environmental exposure; it is always presumed to be work-related unless proven otherwise. Chronic obstructive airways disease (COAD) accompanies asbestosis but may also occur in the context of minimal parenchymal fibrosis and may contribute to accelerated loss of pulmonary function. In some patients, particularly those with smoking-induced emphysema, this may contribute significantly to functional impairment. An exposure history of 10 fibre·years is suggested as the minimum associated with a demonstrable effect on impairment, given available data. Equity issues associated with apportionment include the different criteria that must be applied to different disorders for apportionment to work, the management of future risk (eg. risk of lung cancer for those who have asbestosis), and the narrow range in which apportionment is really useful in asbestos-associated disorders. Apportionment,

*To whom correspondence should be addressed.

attractive as it may be as an approach to the adjudication of asbestos-related disease, is difficult to apply in practice. Even so, these models may serve as a general guide to the assessment of asbestos-related disease outcomes for purposes of compensation.

Key words: Asbestos, Workers' compensation, Apportionment, Epidemiology, Lung cancer, Mesothelioma, Chronic obstructive pulmonary disease, Asbestosis, Pleural plaques, Equity, Exposure assessment, Occupational history

Introduction

Asbestos may cause a variety of health outcomes. Some of these are characteristic but not specific, some are highly specific but uncommon, and some are nonspecific and difficult to attribute[1–3]. Much is known about these conditions, but this knowledge is derived mostly from population studies.

Workers' compensation systems provide insurance for the medical costs of treatment and diagnosis and replace lost income associated with disability resulting from the functional impairment caused by occupational disease. Workers' compensation deals exclusively with disorders arising from occupation or significantly aggravated or contributed to by workplace exposure. Discriminating between occupational and non-occupational causes of disease is fundamental to proper adjudication. It is also necessary in fairness to the interests of employers who fund the system and cannot be held responsible for disorders arising from personal lifestyle, behaviour, or causes unrelated to the workplace. Bringing evaluation down to the individual case is often an ambiguous and uncertain undertaking. However, individual evaluation is essential to the fair adjudication of such cases under workers' compensation. *Apportionment*, which is the estimate of the contribution of a particular cause to the outcome in an individual case, may be a part of this individualized approach[4, 5].

The number of cases attributed to a particular cause in a population is called the *attributable risk* by epidemiologists. The fraction of cases attributed to the cause is called the *attributable fraction*. Attribution, using either measure, is an important public health indicator and may inform the interpretation of workers' compensation claims. However, assignment of attributable risk is an epidemological concept and does not apply to the individual case. Apportionment must be understood always to apply to the individual. For the individual, the attributable fraction is a best estimate only. The fundamental issues of apportionment have been discussed in detail elsewhere[4, 5]. This article will explore the apportionment of cause in asbestos-related diseases where other putative risk factors, such as cigarette smoking, may be present.

Apportionment in Principle

In almost all Canadian jurisdictions, workers' compensation boards are required to accept claims in their totality if a substantial component of the disease is work related. However, defining what constitutes a substantial, significant, or minimal component is often difficult. A possible alternative approach is apportionment, which some boards have already used on a relatively informal basis to allocate responsibility for claims.

Workers' compensation boards in all jurisdictions are faced with an expanding challenge in the management of claims related to occupational disease. Questions of causation, the presence of multiple risk factors, and modifications of the characteristic presentation of occupational diseases greatly complicate adjudication.

Asbestos-related diseases are particularly problematical in this regard and illustrate these problems well. Among these fundamental issues is the relative contribution of different causes, such as cigarette smoking or asbestos exposure, to the risk of a disease such as lung cancer or to overall impairment from on outcome, such as chronic obstructive airways disease. It is generally easier to distinguish occupational from nonoccupational disease when characteristic outcomes are specific to the exposure, as occurs with pneumoconioses such as asbestosis or when the association is so great that a presumption is reasonable, as in mesothelioma. However, when the outcomes are not specific, and especially when they may also be caused by other common environmental exposures such as cigarette smoking, defining causation can be problematic.

Causation may be reduced, in most cases, to a proposition of "but for", a term commonly used in law. If "but for" exposure to the hazard, the condition would probably not have occurred, the hazard can be considered to be the cause. Another way of saying this is that the cause was necessary, even if it was not sufficient. Applied to asbestos-related

disease, assessing that the possible causes include asbestos exposure at a level that may have substantially contributed to disease is the first step. The second would be to assess the relative contribution of asbestos compared to other causes, the step called apportionment.

Apportionment by cause

The process of adjudicating workers' compensation claims involves a differentiation between occupational and nonoccupational causes of disease and injury. Though in practice this can be exceedingly difficult, and in some cases impossible, the requirement to consider causation is fundamental to the philosophy of workers' compensation. That is because workers' compensation systems are mandated to resolve individual claims on the best evidence, not to generalize to groups or classes.

Faced with a large number of difficult occupational disease cases, workers' compensation agencies have considered apportionment by cause. Apportionment by cause is the estimation in an individual case of the relative contribution to an outcome, such as a multi-factorial disease, of several risk factors or potential causal exposures that are present in the case and that are known to be associated with the outcome. Apportionment by cause is a way of apportioning responsibility and contribution to the final outcome. In workers' compensation, it principally applies to apportioning causation between occupational and non-occupational risk factors.

There are other ways to apportion. Apportionment of impairment and disability, for example, is common in multiple injury cases. In the tort system, the equivalent concept is apportionment of harm (meaning responsibility for causing harm) but because workers' compensation is a no-fault insurance system the assignment of blame or responsibility is not so useful.

Apportionment by cause must be performed on the individual case. Individuals may vary in their characteristics from the population as a whole. Often, apportionment cannot be determined with certainty and epidemiological data may then be used to derive an estimate of the relative contribution of a risk factor in an individual claim. However, this must be understood to be a derived estimate, not to be confused with attribution, which uses the population attributable fraction, or the apportionment of impairment or its social derivative, disability, which can be done by specific measurement in the individual case.

The benefits of fair and accurate apportionment are obvious: adjudication may be simpler, adjudication may be fairer to employers and some injured workers and financial resources would be conserved for workers with greater impairment. Workers might be encouraged to take responsibility for their own health, fiscal exposure would be more fairly shared among health care funding agencies and the relative contribution to disability benefits for permanent impairment could be divided among payers, such as provincial health care plans, Social Security or Canada Pension, and workers compensation. Although apportionment is an attractive option for adjudication in compensation, it has many drawbacks and uncertainties. These are explored in detail elsewhere[4, 5].

For apportionment to work in practice, two related concepts must be introduced: presumption and substantial contribution.

Presumption

A presumption exists when a worker with a compatible exposure history develops a particular disease and the condition is assumed to be related to the exposure. The principle of presumption requires that the disorder be sufficiently common among workers with that exposure that in any given case it is more likely than not that the disorder is work-related. The logic of presumption requires that a risk attributed to exposure in an exposed population must equal or exceed double that of people without exposure, because a relative risk of two corresponds to even odds which corresponds to the legal requirement of "more likely than not", all other things being equal. A rebuttable presumption is one that can be challenged on the particulars of the case, for example when the claimant or plaintiff had not accumulated sufficient exposure to expect a substantial contribution.

Substantial contribution is, simply, the requirement that a claimant have been exposed to a sufficient quantity, concentration or duration of exposure of the hazard, in this case asbestos, to cause at least a minimal injury that could contribute to the outcome. This is not quite the same as a threshold because a threshold may be defined in various ways. As a practical matter, the purpose of the requirement for a substantial contribution is to reduce the number of claims without real merit and to increase the likelihood that those claims remaining are associated with work-related exposures and are therefore apportionable.

One may propose the following essential criteria for a definition of substantial contribution:

- The contribution to the outcome (regardless of the subsequent impairment) should be demonstrable in some way or inferred from population data; a history of nominal exposure or the presence of a marker that does not correlate with risk is not enough.

- The contribution should be on the same order of and significant relative to natural individual variation and the loss of function in progression of disease.
- For example, if the normal adult change in FEV_1 is –30 ± 7 ml/year and –60 ± 10 ml/y is associated with chronic obstructive pulmonary disease by age 60, an additional incremental loss of 10 ml/y due to an occupational exposure would clearly be significant (representing one-third of the contribution leading to pathology) but 5 ml/y would not so clearly be significant, because it falls within the range of measurement error and normal variation. In practice, the "noise" in measurement and lack of baseline measurements may make this difficult to apply.
- In cases where impairment results from loss of function due to the disease outcome, the proportion of impairment contributed by the cause in question should be enough to change the prognosis or clinical course; in other words, enough to make a difference in a borderline case.
- Whatever the contribution to the outcome, it should plausibly relate to the permanent impairment; in other words, if the presence of a pleural plaque does not predict airflow obstruction, demonstration of a pleural plaque cannot be used to suggest a substantial contribution of asbestos to causing airflow obstruction, notwithstanding their association with a restrictive component of reduced ventilatory capacity[6].

One approach to defining substantial contribution is to identify a level of exposure commonly associated with definite functional changes that may be of significance in the progression of disease. In the real world of workers' compensation, detailed exposure information over the lifetime of the worker is simply not available. More robust approximations are needed. In practice, this may mean resorting to general or approximate categories.

When there is a possibility of error, workers' compensation policy is almost always to give the benefit of the doubt to the worker. Usually this is written into the legislation creating the workers' compensation system. Estimates of substantial contribution should therefore be set at a level that will include all or almost all claimants who are likely to be affected by their exposure. The tradeoff is to be less efficient to exclude as many as possible of claimants who are not likely to have been affected, erring on the side of inclusion.

Asbestos-Related Disease

Occupational disease claims, including asbestos-related cases, tend to be complicated and less certain than occupational injuries. Asbestos-related claims may be more amenable to adjudication than occupational asthma but remain open to interpretation and subject to assumptions that are difficult to prove. In a detailed study of the handling of claims by Washington state in the period 1982–1986[7] for a high-risk population in which occupational disease had been diagnosed at a university-affiliated clinic, only half of claims in the state system were accepted and there were suggestions of bias in the adjudication against nonwhite claimants and by adjudication system. Criteria for acceptance were inconsistent among systems and within the state system; there was no or unexpectedly low correlation between claim acceptance and chest film (ILO category), presence of restrictive changes, smoking status, or concurrent obstructive lung disease. Other, older studies have shown similar findings (cited in 7).

More recent studies suggest that in British Columbia and possibly Australia only about 10% of asbestos-related lung cancer cases have been recognized and compensated appropriately[8]. A high mortality from potentially asbestos-related disease, including asbestosis, has been reported among workers potentially eligible for compensation in Ontario. These workers also often did not file claims[9]. The problem appears to be not one of acceptance but of the claims not having been filed in the first place.

Chrysotile

In this discussion, no distinction will be made between chrysotile and amphibole forms of asbestos, except as noted. Although there are apparent differences with respect to potency for different outcomes, some risk is present for all forms and these differences play little role in apportionment[2, 10].

Chrysotile has been the leading form of asbestos used for industrial insulation in the Americas and the UK and the experience reflected in epidemiological studies of end-users, such as insulators, reflects predominantly chrysotile exposure. Insulation is the source of exposure of greatest concern in Japan, as elsewhere. Most of the asbestos on which the earlier insulators studies were conducted were also associated with chrysotile exposure, mostly from Quebec. Some of the highest risk estimates reported in the asbestos industry (e.g. the South Carolina textile plant) were in fact associated with chrysotile exposure (without obvious contamination by tremolite)[11, 12]. The conclusion is inescapable: chrysotile is itself a cancer hazard[10].

The data on chrysotile-associated risk among Quebec asbestos miners is irrelevant. It is true that many of the studies used to calculate risk estimates for exposure to chrysotile reflect the exposure of miners and mining

communities. However, miners consistently show less risk than would be predicted based on the experience of end-users, such as insulators. This is so consistent that it is now generally accepted that the experience of miners is a poor guide to the assessment of risk, probably because fibre size and degradation to fibrils is less advanced in mining and refining and further advanced in manufacturing and application of insulation. Although chrysotile may be less potent than other forms of asbestos for most outcomes, it is still hazardous and responsible for the observed health effects[11, 13].

Chrysotile and amphiboles

Chrysotile has been contaminated with amphibole forms of asbestos, especially with tremolite, in the past. Some investigators believe that the small residual amphibole content of chrysotile asbestos is responsible for the cancer risk associated with chrysotile-exposed workers. Even if this were true, the outcome would still be work-related and therefore compensable. The end users described above, especially insulation workers, generally used products in which amphibole contamination was not likely to be a major factor. The entire issue is therefore irrelevant for purposes of compensation management[13].

Bronchogenic Carcinoma

Lung cancer is the most difficult problem in apportionment problem among asbestos-related diseases[14]. There are many causes of lung cancer, many of them occupational, and one major lifestyle cause, cigarette smoking. Apportioning between occupational and nonoccupational causes of lung cancer in a worker exposed to asbestos, therefore, is almost always an issue of ruling out the significance of other occupational exposures and then estimating the most likely contribution of asbestos against that of cigarette smoking.

Smoking and asbestos exposure

Complicating matters is the fact that there is a positive interaction between asbestos exposure and smoking in conferring risk of lung cancer. In the classic studies conducted on insulation workers and other groups in the 1970's, it was observed that asbestos exposure alone conferred a risk of lung cancer approximately 5 times the baseline risk of a nonsmoking person not exposed to asbestos. Cigarette smoking alone conferred a risk approximately 10 to 15 times that of the baseline. However, the combination of work-related asbestos exposure and cigarette smoking was associated with a risk of 50 to 100 times the baseline, far greater than if both risks were simply added, and roughly what one might expect if they were multiplied, and provides a classic example of multiplicative (synergistic) interaction.

This interaction reflects an underlying biological mechanism. This mechanism clearly acts to amplify the effects of the exposure to asbestos to greatly enhance the risk following combined exposure and does so in a non-linear fashion. This means that it is not possible to trade off the effects of asbestos and smoking as if their contributions were additive, or linear. Because the risks of lung cancer are nonlinear, simple regressions or calculations of relative risk associated with a given level of asbestos exposure and a given smoking history cannot resolve the problem. A much more complicated interactive regression, or curvilinear function, would be required to estimate the contribution of each factor. In practice, an attempt to apply such a complicated formula based on statistical patterns in a large population, with large variance, would appear arbitrary in the case of an individual and would be open to challenge based on the characteristics of the individual claimant.

One problem in dealing with this interaction is that past studies of lung cancer among smoking asbestos-exposed workers were based on much higher asbestos exposure levels than occur today, and were documented in populations with a generally higher prevalence and intensity of smoking than occurs today. (They also did not break down this observed interaction by age group, which would be helpful in thinking about apportionment.) The old rules of thumb may no longer apply in an era when asbestos exposure is far less, with concomitant reduction in cigarette smoking. As the magnitude of each exposure is reduced, it is likely that the interaction becomes less as well, because it too is likely to be exposure-dependent. Thus, one must conclude that although the apportionment by cause of a lung cancer to asbestos or cigarette smoking is not a simple linear tradeoff, it is probably no longer a tradeoff between steeply exponential curves either. Paradoxically, this reduces the influence of cigarette smoking as the dominant factor in the equation and makes it easier to conceptualize a tradeoff between the two factors.

At first, it might seem that because cigarette smoking accounts for most of the risk for developing lung cancer, the odds that a cancer was caused by cigarette smoking in a person who smoked but was not exposed to asbestos was 10 to 1. Applied as an estimate of apportionment in someone who only smokes, this results in 90% apportionment by cause. This leads to a clearly justified presumption that in all cases of comparable smoking history a lung cancer would have been caused by the cigarette smoking. Correspondingly,

the odds that a cancer was caused by asbestos in a person who was exposed but did not smoke would be 5 to 1, clearly justifying the presumption in a nonsmoker. If the tradeoff were linear, it might be tempting to compare the tenfold risk against the fivefold risk and to conclude that cigarette smoking was twice as important a factor, for odds of 2 to 1.

However, this is not logical in the context of workers' compensation. It does not take into account the interaction or modification of risk between cigarette smoking and asbestos. Because employers, or government regulations, did not or could not ban smoking among their employees, both on and off the job, as a condition of employment, they must "take the worker as they come". The preferred analysis would be to observe that risk is excessive *among smokers*. This is the only relevant comparison if one "takes the worker as he (she) comes" and applies the "thin skull" rule, that unusual susceptibility in the injured party does not absolve the tortfeasor of liability (In workers' compensation, of course, the employer is not held liable. The principle merely shifts the burden of liability to the system to accept the claim.).

The rules of rebuttable presumption remain useful in this application. The evidence suggests that in the majority of cases, the risk of lung cancer in an asbestos-exposed smoker is more than double that of a smoker not exposed to asbestos. If so, then *among smokers* it is more likely than not that "but for" the asbestos exposure the exposed worker would not have developed the cancer. This applies the usual legal test for causation. The odds that a cancer was associated with asbestos exposure in a cigarette smoker compared to a nonexposed cigarette smoker would then be around 5 or 10 to 1. This is more than enough to justify a presumption that in any smoker exposed to asbestos, the cancer in question was due to the asbestos exposure.

The fact of smoking increases risk for the worker but it also increases the potential effect of asbestos exposure. "But for" the asbestos the probability of the individual smoker developing the lung cancer would have been much less. Not even a positive interaction between asbestos exposure and cigarette smoking is required to justify a presumption on this basis, as long as the combined risk is at least double that of cigarette smoking alone.

Given this analysis, it is clear that in either smokers or nonsmokers, the occurrence of bronchogenic carcinoma in a worker exposed to asbestos at a substantial level should be apportioned 100% to the asbestos exposure. The issue of apportionment in lung cancer should therefore become a rebuttable presumption.

"Substantial contribution" in lung cancer

An index of exposure is required to separate claims for lung cancer that may have an association with asbestos exposure from those that probably do not. This derivation applies only to risk of lung cancer and is consistent with levels used for purposes of settlement in a class-action suit in the United States.

We have previously applied[15] a quantitative risk assessment of exposure to airborne asbestos in an office building, based on a simple mathematical model developed by Hughes and Weill[16]. This model is consistent with that used for asbestos-related claims adjudication by the Central Claims Facility (CCF) in the U.S. We now have adapted this model with a slightly different derivation and have adjusted assumptions to conform to the group of asbestos workers showing the highest risk for lung cancer (asbestos textile workers). These are very conservative assumptions, meaning that no asbestos worker who develops lung cancer as a result of asbestos exposure is likely to be omitted but that some who develop lung cancer unrelated to asbestos exposure will be accepted.

The derivation is as follows:

O = observed cases, E = expected number of cases,

SMR = standardized mortality ratio ($O/E \times 100$, equivalent to relative risk expressed as a percentage),

B = slope of the linear extrapolation of the incidence curve related risk of lung cancer to cumulative asbestos exposure expressed in fibres per cubic centimeter per year (this is adapted from Hughes and Weill[16] and equals 'b/100' in this equation. We used b/100 because it was more logical and to separate out 'd';

d = total cumulative dose (in terms of fibres/$cm^3 \times$ years, the terms *presumably* convertible to fibre-years if ventilatory volume and clearance could be accounted for).

The derivation of a reasonable "threshold" exposure for substantial risk is governed by the equation of Hughes and Weill[16]:

$$\text{Excess deaths} = O - E = EBd$$

For purposes of legal criteria, we are interested in the risk level at which it is "more likely than not", giving benefit of doubt to claimant, that a lung cancer is associated with asbestos exposure. This risk level compounds to even odds, a relative risk of 2.0, a relative attributable risk of 1.0, and an SMR = 200.

Therefore: $0 - E = 2E - E = E = EBd$

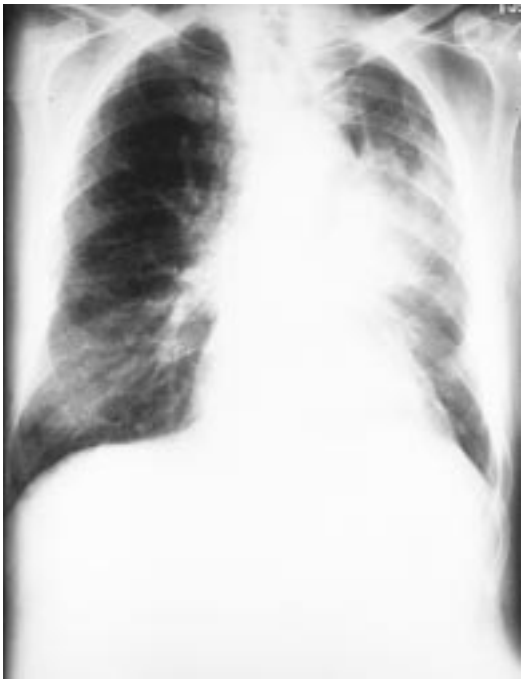

Fig. 1. Bronchogenic carcinoma in an asbestos cement pipe worker, against a background of asbestosis.

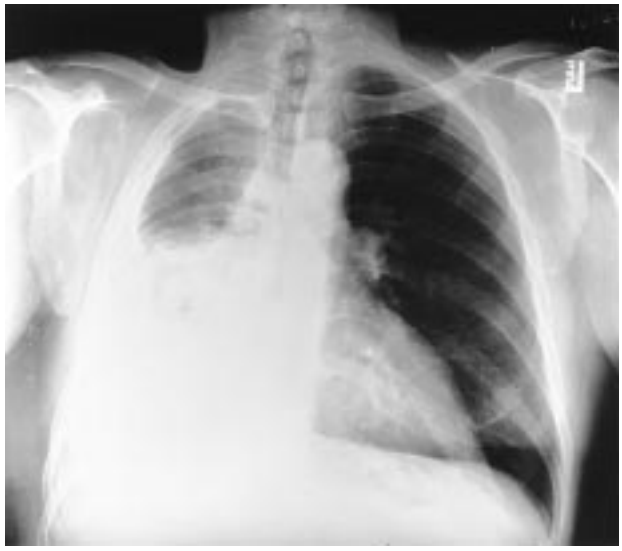

Fig. 2. Mesothelioma in another asbestos cement pipe worker, with no radiographic signs of asbestosis.

The value of 'B' is taken from Fig. 1 of Hughes and Weill[16], B = 0.025 (in inverse units of f/cm^3·y) and from the *highest* risk group (textile workers):

$$d = 40 \text{ f/cm}^3\cdot\text{y}$$

This means that any combination of fibre exposure and duration of employment that yields this rate for 'd' will correspond to a legal definition of "more likely than not" + benefit of doubt.

Translated into terms of duration of employment, this means:

- 8 years at 5 f/cm^3·yr, consistent with CCF high risk group
- 10 years at 4 f/cm^3·yr, consistent with CCF intermediate group
- 15 years at 2.7 f/cm^3·yr, consistent with CCF low risk group.

If an individual shows a mixed employment history, moving among occupations in different risk categories, one may apply a very simple weighting system as follows:

- high risk occupations: count 1.25 years of eligibility for every year of employment
- intermediate risk occupations: count 1.00 years of eligibility for every year of employment
- low risk occupations: count 0.67 years of eligibility for every year of employment

Although occupational histories may be only approximate in reflecting level of exposure, recent studies suggest on acceptable correlation for this type of classificiation[17].

This set of criteria is actually relatively conservative compared to other jurisdictions. The German "Berufgenoßenschaften" (workers' compensation panels) have recently adopted a threshold of 25 f/cm^3·y for accepting claims in that country (Information supplied by the International Labour Organisation.). This is a widely accepted "threshold" estimate (not a true toxicological threshold) originally proposed by the Royal Commission on Matters of Health and Safety Arising from the Use of Asbestos in Ontario[18].

Clinical markers of substantial contribution

Although this article is primarily concerned with apportionment, the issue of causation in asbestos-associated lung cancer requires further attention. Fundamentally, this is a problem of identifying markers of effect that suggest that the claimant was exposed at a level that makes a substantial contribution to risk. As a practical matter, the markers of greatest interest have been radiological, the early identification of fibrosis and the role of pleural plaques.

For many years there has been a dispute over whether asbestos-associated lung cancer can occur in the absence of interstitial fibrosis and early asbestosis. This has resulted in a great deal of confusion[19, 20]. However Churg and Green[18] have argued persuasively that fibrosis is a necessary concomitant of asbestos-related bronchogenic cancer risk.

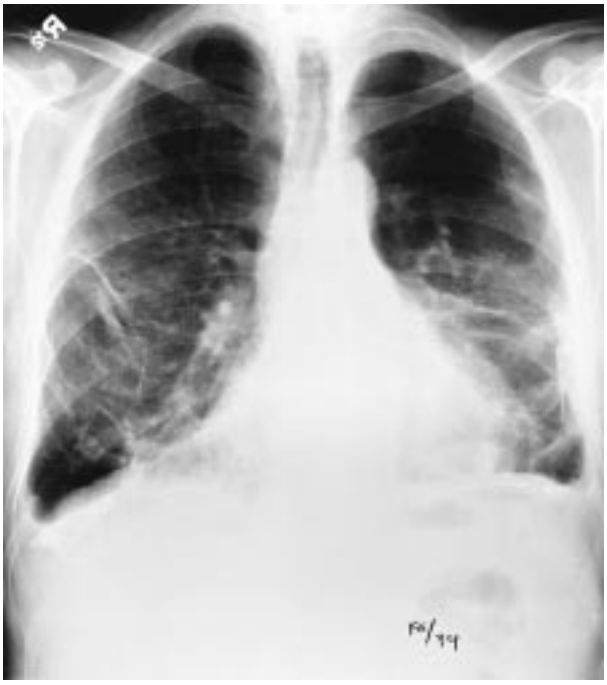

Fig. 3. Chest film showing classical features of asbestosis: irregular opacities, fibrotic bands, interlobar fibrosis, blunted costo-phrenic angles, diaphragmatic tenting and plaques, pleural plaques, shaggy heart border, mediastinal displacement and parenchymal nodule.

The clinical and medicolegal issue is how much fibrosis is required for risk to be demonstrated and can this level of fibrosis be detected by routine clinical tests[21].

Recently, a major paper by Weill[22], following up on earlier findings by Hughes and Weill[23], suggested that among asbestos cement workers who had 20 or more years of experience, only those with category 1/0 disease or greater involving small irregular opacities on their chest film (by the ILO classification of the pneumoconioses) were at risk of lung cancer. This article was widely interpreted as suggesting that some degree of early asbestosis was necessary to conclude that the degree of asbestos exposure was sufficient to be associated with an excess risk of lung cancer.

However, this is a flawed interpretation. Category 1/0 is not clear evidence of disease and is just over the boundary from a nominally normal film. There is no "bright line" boundary between 0/1 and 1/0, only an interpretation of profusion that differs in degree. Lung content of asbestos fibres shows a continuous trend from low levels at 0/0 progressing through 0/1 and 1/0 to 1/1, not a clear threshold. Since there is no threshold for asbestos exposure and risk of lung cancer, one would not expect an arbitrary threshold for risk associated with category 1/0 profusion. Finally, Weill did not explain how workers who had gone that long exposed to asbestos without developing 1/0 profusion may have differed from those who did; it may be possible to develop up to a 0/1 film on the basis of cigarette smoking alone and cigarette smoking accelerates the appearance of opacities among asbestos-exposed workers[24, 25]. For all these reasons, this study is not definitive in suggesting that changes compatible with interstitial fibrosis are necessary to accept a lung cancer as asbestos-related, although it has been so interpreted (Weill's major point in the paper was actually that the mechanism of lung cancer is associated with the alveolitis that occurs as the first pathological event in asbestosis.).

An equally careful study by Wilkinson *et al.*[26] demonstrated that asbestos-exposed workers with category 0/1 or 0/0 (normal) films had an increased risk of lung cancer compared to workers who had no history of asbestos exposure, regardless of film category. The risk was less than that of asbestos-exposed workers with 1/0 changes, with odds ratios of 1.56 and 2.03, respectively. In their data, the association was clearly present, it was statistically significant, and it was dose-dependent, with the chest film category presumably crudely indicating dose.

One reasonable interpretation of Wilkinson *et al.*[26] is that it supports the idea that a chest film of 1/0 or greater is needed for the presumption of lung cancer as asbestos-related but that chest films at 0/1 do not exclude asbestos as a cause. Chest films classified as 0/0 suggest that an association between lung cancer and asbestos exposure is less likely but cannot rule out such an association.

Histological studies tend to confirm this interpretation; in a significant proportion of cases of lung cancer in asbestos-exposed workers, parenchymal fibrosis is not visible on the chest film[27]. Histological or microscopic interstitial fibrosis also may not be a necessary concomitant of asbestos-related lung cancer. Individual studies have suggested that asbestos-related bronchogenic carcinoma is "almost always" associated with histological asbestosis but have also demonstrated a relationship between degree of fibrosis and risk that is compatible with an excess risk at lower levels of fibrosis, below 1/0[19, 28]. Egilman and Reinert[16] reviewed the available evidence for an association between fibrosis at the tissue level and lung cancer (as they did for a clinical or radiographic correlation) and concluded that although several different studies used rather different approaches and methods, they were consistent in suggesting that there was only a statistical association reflecting the history of asbestos exposure. They concluded that although workers exposed to asbestos were more likely to have fibrosis at the

time of resection or death from lung cancer, many asbestos-exposed workers with lung cancer did not have microscopic fibrosis, occasionally despite greatly elevated fibre burdens. They suggest that the alveolitis that results in fibrosis and that probably predisposes to lung cancer is not invariable and that epithelial metaplasia and proliferative fibrosis do not necessarily occur together or stepwise in progression, although both may be caused by asbestos fibres.

Egilman and Reinert[19] do not address the issue of whether the cases in which this association does not occur at necropsy might just represent "background" lung cancers not associated with asbestos. However, they cite individual studies that suggest that this is not the case. On a group basis these cancers were more frequent and more likely to be distributed in the lung in areas likely to be affected by asbestos (for example, in the lower lobes) compared to persons who were not exposed to asbestos[6]. If histologically demonstrable asbestosis is not associated with lung cancer, then advanced methods for detecting early asbestosis[29] such as HRCT[30] would not be useful either in ruling out an association with asbestos either but are valid markers of past asbestos exposure.

Pleural plaques are also not satisfactory predictors of asbestos-related lung cancer. Weiss[31] has critically reviewed this literature and has pointed out the methodological limitations in all extant studies. However, for the purposes of apportionment a more useful question is whether workers who develop lung cancer are more likely to have pleural plaques than asbestos-exposed workers who did not develop cancer. Unpublished data from Hughes cited by Weiss[31] describes an odds ratio of 1, suggesting that the presence of pleural plaques cannot be used as a marker to associate lung cancer causally with asbestos exposure. Subsequent studies[32] and a more recent review[33] have not changed this conclusion.

It is often difficult to demonstrate asbestos fibres in cases of lung cancer, even with a clear history of exposure to asbestos[27]. For this and other reasons related to under-recognition, British Columbia investigators[8] have concluded that asbestos-related lung cancer is substantially underrecognized in both Canada and Australia and that as many as 90% of cases may be missed.

The most reasonable conclusion with respect to apportionment among cases of lung cancer in asbestos-exposed workers appears to be to treat the association as a rebuttable presumption. If there is a confirmed history of exposure to asbestos, neither pleural plaques nor parenchymal fibrosis is required to demonstrate sufficient exposure. If the British Columbia investigators are correct, fewer cases will be misclassified by a presumption than by rigorously enforcing the requirement for objective evidence of an asbestos-related effect. Obviously, that policy would require acceptance of many more claims, raising the question of setting limits.

Mesothelioma

The most dread outcome of asbestos exposure is mesothelioma, a cancer with a poor prognosis and an almost invariable association with asbestos exposure. Mesothelioma in the presence of a history of asbestos exposure must be presumed to have been caused by asbestos. Chrysotile asbestos is generally considered less likely to induce mesothelioma than amphibole forms[34]. In practice, even a history of exposure to chrysotile alone does not rule out an association because of contamination or concomitant use of amphiboles. Cigarette smoking does not increase the risk of mesothelioma and there is no evidence that it modifies the clinical course or progression of the cancer.

Thus, any impairment associated with the cancer, including pain, chest wall mechanical problems, respiratory insufficiency, and disabling symptoms, are apportioned entirely to asbestos. Given the poor prognosis for recovery, the subjective symptoms that will accompany progressive impairment, and the conversion of these realities into reduced capacity to work and to disability, it is only reasonable to apportion both cause and impairment to the asbestos as soon as the symptoms or signs of mesothelioma become manifest. Both the original impairment and the prognosis for permanent impairment are soon determined by the tumour, and the cause of the mesothelioma can be presumed in almost all cases to be the asbestos exposure.

Asbestosis

Asbestosis is the characteristic pneumoconiosis associated with inhalation of asbestos fibres. The term should never be used generically to refer to asbestos-related disorders, as this leads to unnecessary confusion[36].

Like all pneumoconioses, asbestosis as a process consists of the direct effect of the dust, and also of the effect on the lung of the reaction to its presence. In asbestosis the pulmonary response is exuberant fibrosis, occurring in parenchyma (alveolar region) of the lung, initially adjacent to the airways in response to an alveolitis, or inflammation of the airspaces. Early asbestosis resembles the disease known as usual interstitial pneumonia (UIP), a synonym for fibrosing alveolitis and idiopathic pulmonary fibrosis. Indeed, there is a hereditary form of UIP that may conceivably

place some workers at risk for fibrotic lung diseases such as asbestosis, but this has not been adequately studied.

Characteristic of both early asbestosis and UIP is the presence of an inflammatory reaction that can be measured by bronchoalveolar lavage (BAL), in which cells and secretions from the deep lung are obtained by bronchoscopy. With advancing disease the fibrosis becomes more extensive, and is more likely to be associated with other asbestos-related changes in the thorax. The diagnosis of asbestosis is usually made on the chest film, but computerized tomography (CT) and high-resolution computerized tomography (HRCT) are increasingly used to establish the diagnosis[36]. Both are more sensitive than conventional chest radiography in identifying interstitial fibrosis[1].

The final common pathway for both asbestosis and UIP, and for a variety of other pneumoconioses, is a coarse pattern of parenchymal fibrosis called honeycombing. Asbestosis is characterized by the presence of asbestos fibres and asbestos bodies, which distinguishes the condition from UIP and other fibrogenic pneumoconioses. Asbestos bodies are much easier to see, but are much less common than asbestos fibres. New cases of asbestosis in recent years have usually not been so severe as in the past, when honeycombing and fibrous bands were common in advanced asbestosis cases. Fibres from tissue recovered at autopsy or biopsy were sometimes difficult to visualize because of the mass of scarred tissue, but total fibre counts from ashed tissue were very high in such cases[20].

The fibrosis associated with asbestosis rarely occurs in complete isolation. More commonly it is associated with a variety of asbestos-related changes in the thorax that are more or less characteristic of asbestosis as a disease and are seen only rarely in other conditions. These include:

- pleural fibrosis with diffuse and circumscribed plaques, especially on the diaphragm;
- progressive loss of definition of other structures in the thorax, especially the heart; bullae (large thin-walled holes in the lung);
- asbestos-associated cancers (often difficult to see by chest film in the fibrotic lung); and
- distortion of organs in the mediastinum.

These secondary changes are now uncommon because exposure levels are substantially lower, and are unlikely to produce such extreme manifestations of disease.

The process of fibrosis in asbestosis is relatively localized to the interstitium (the structural connective tissue in the lung that lies between alveoli) and over time becomes thicker and more diffuse. Initially the fibrosis begins as isolated patches that coalesce into rough or spiky-shaped masses that appear as irregular opacities on a chest film. These opacities are most frequent, and therefore most dense, on the chest film in the lower lung fields. Over time, they tend to coalesce into larger masses or opacities and may sometimes present as nodules, in which case cancer must be ruled out, or as bands of fibrosis. Ultimately, the scarring may become gross and interfere with the mechanical function of the lung.

In asbestosis the airways are also affected but not as much as the parenchyma. Pulmonary function studies may show a mild obstruction to airflow, particularly early in the course of the disease[36, 37]. In more advanced or rapidly progressing cases of asbestosis, this obstructive component is usually soon overwhelmed by a progressive restrictive disease, at least in part due to air trapping[38] that limits the capacity of the lungs and that ultimately may cause respiratory insufficiency. In less advanced or progressive disease, there is an accelerated loss of ventilatory capacity, sometimes appearing before radiographically evident asbestosis. In such cases, however, the progression of the chronic airflow obstruction is greater with greater profusion of irregular opacities on the chest film[39]. The apportionment of chronic obstructive airways disease as an outcome of asbestos exposure is discussed in a later section. Combined restrictive and obstructive deficits in asbestos-exposed workers seems to be associated with greater functional impairment[40].

Because it is difficult to appreciate obstructive disease against a background of severe restrictive disease, the airways component of asbestosis has not received much attention until recently. Pleural fibrosis is particularly associated with these restrictive changes and probably represents the contribution of mechanical changes in the chest wall, but this is a relatively minor effect[41–43]. Pulmonary function studies also show a reduced diffusing capacity, both because of delayed diffusion across the thickened interstitium and mismatching of blood and air in the alveolar region due to the disruption of the fibrosis. This mismatching is also a reason for the progressive desaturation of oxygen in the blood that eventually results in hypoxemia and clinical respiratory insufficiency in severe cases. Mild cases of asbestosis may not necessarily show this interference with gas exchange and blood gases may be normal in such cases.

Unlike other outcomes associated with asbestos, there is no evidence that cigarette smoking plays any role in contributing to the onset of asbestosis, or that the effects of asbestos exposure and cigarette smoking are positively interactive in causing enhanced asbestosis[44]. There is some evidence that once established, asbestosis may be enhanced by cigarette smoking with an increased frequency of opacities

detectable by HRCT for the same degree of asbestos exposure[45]. Since the frequency of opacities does not correlate closely with changes in pulmonary function and therefore impairment, it is not clear that this finding can be used as the basis for an apportionment formula.

Possible susceptibility states may contribute to risk of asbestosis, for example glutathione-S-transferase deficiency[46]. This is a common condition, affecting some 50% of Caucasian males, that might well be considered within the range of normal but that appears to predispose to asbestosis and may modify the outcome. However this observation is not helpful in apportionment. It is an inborn condition of the worker and so common that it may be considered a variant of normal.

The implications of these data simplify apportionment in most cases. Because asbestosis is a disease only caused by exposure to asbestos, and because other risk factors play only a minor role in modifying the outcome associated with the fibrosis (as opposed to complications such as cancer), there is no basis for apportionment by cause. If the diagnosis is asbestosis and causation can be established, the apportionment by cause is 100% attributable to asbestos and all respiratory impairment resulting from the fibrotic component of the disease is asbestos-related. Examiners often acknowledge the presence of asbestosis, but apportion the resulting respiratory impairment between asbestos and cigarette smoking, particularly when there is mixed obstructive/restrictive impairment. It is difficult to do this by cause for the obstructive component and the progression of mixed impairment makes separation of the restrictive and obstructive components uncertain. Given the caveat in workers' compensation that any substantial contribution by a workplace exposure is sufficient to consider the outcome to be work-related, the presence of any documentable asbestosis-related impairment, for example mild restrictive impairment, should be sufficient to apportion all impairment to the asbestos exposure.

The general rule that in the presence of asbestosis all respiratory impairment should be apportioned to asbestos. The exception may be a very mild case of asbestosis with minimal or no functional impairment associated with marked obstructive changes in a heavy smoker, a characteristic smoking-related respiratory impairment. In such a case, the restrictive component of the disease would be considered asbestos-related and the obstructive component, taken as FEV_1/FVC(%) rather than FEV_1 compared to predicted, would be more likely to reflect the influence of cigarette smoking. The treatment in such a case would then parallel that given below for chronic obstructive airways disease. However, even in this case there is evidence that the asbestos-related airways changes modify the effects of cigarette smoke, at least in experimental studies[46, 47]. The relative contribution by cigarette smoking may be overestimated by this approach in such cases.

Chronic Obstructive Airways Disease

It has been known for many years that exposure to asbestos is associated with obstruction to airflow as well as restrictive changes[50–52]. Functional changes are also correlated with respiratory symptoms such as cough, wheeze, and shortness of breath[53]. However, chronic obstructive airways disease (COAD) has not been emphasized as an asbestos-related outcome and has not been accepted by compensation agencies as a presumption or scheduled occupational disease. There are several reasons for this reluctance to recognize asbestos-related chronic obstructive airways disease. The most influential has probably been that the effect of cigarette smoking is not easily separated from asbestos exposure and has confounded the association, influencing agencies and adjudicators to attribute all of the cause to the smoking[50]. Another factor is that the predominant effect in advanced asbestosis is restrictive disease and the obstructive changes associated with lesser degrees of asbestosis have been largely overlooked[37, 39]. Yet another factor is that mandated surveillance for asbestos-exposed workers, such as the OSHA asbestos standard in the United States and the Alberta Fibrosis Program in Canada, have emphasized the early identification of restrictive changes and changes in the FEV_1, which will reflect changes in the FVC, rather than an interpretation that emphasizes airflow taking changes in vital capacity into account.

Adults lose a fraction of their lung capacity and airflow velocity, as measured by routine spirometry, due to aging; this loss is predictable, and for FEV_1 averages 30 ml/y. In theory, any person who lived long enough would develop obstructive disease, once the natural loss progressed far enough. Pulmonary injury may accelerate this loss and in cigarette smokers this rate of loss may easily double or triple, so that during their lifetime they dip well below the normal range and develop incapacity, the condition known as chronic obstructive pulmonary disease (COPD). (COPD and the less common term COAD are usually synonymous. Here, COAD is the more general term, and is used to avoid confusion with the complex illness associated with cigarette smoking that most clinicians have in mind when they refer to COPD.)

It is now well established that asbestos-exposed workers

show accelerated loss of airflow and are at risk for obstructive airways disease[54–59]. Those with signs of early parenchymal fibrosis appear to be at higher risk for more rapid decline[60]. Asbestos-exposed workers who develop persistent respiratory symptoms are at risk for even more rapid loss of pulmonary function[61]. There is also experimental evidence for a positive interaction (synergy) in airflow obstruction between asbestos exposure and cigarette smoking because of changes in compliance in the wall of small airways[47].

Studies of nonsmoking asbestos-exposed workers confirm that asbestos exposure alone can accelerate loss of pulmonary function[36, 54, 55, 62–64]. The two studies that permit inference of the rate of loss of FEV_1[55, 64] suggest that the accelerated rate of decline, over the usual 30 ml/y, is on the order of 30 to 60 ml/y or a doubling or tripling of the normal rate. The decline in FEV_1 was greater with higher exposure levels. This is in the same range as the effect of cigarette smoking.

The pathology and physiology of this effect is reasonably clear. The alveolitis induced by asbestos begins at the respiratory bronchiole, which is anatomically adjacent to the terminal and other small bronchioles. As well, there may be direct inflammation of the bronchiolar wall in response to deposited asbestos fibres[47, 65]. The adjacent alveolitis changes the compliance of the wall of the small airways (which is membranous, unprotected by cartilage) and, together with loss of the elastic recoil of the surrounding lung parenchyma, causes a progressively larger fraction of the population of small airways in the lung to close earlier on expiration, trapping air and introducing resistance to airflow. Asbestos therefore causes a small airways disease that appears first as reduced flow rates in the mid-expiratory part of the spirogram, which reflects airflow in the small-diameter but high-cross section peripheral airways, where there should normally be very little resistance to flow. This may occur with or without early signs of asbestosis[36]. Šaric and Peric[66] have proposed that this process follows an initial phase of several years in which small airways airflow actually increases due to stabilization of the bronchiolar wall by fibrosis.

Cigarette smoking induces a focal bronchiolitis and minimal adjacent alveolitis in much the same way. Over time, a loss of elastic recoil, early collapse of the bronchiole, and small airways disease ensues. An important component of this process, also presumably critical in asbestos-related bronchiolitis, is the release of inflammatory mediators and protease enzymes that degrade structural protein, which result in local tissue destruction. This chronically progresses to overt emphysema. To date, there is no evidence for interaction between cigarette smoking and asbestos as a cause of small airways disease or loss of FEV_1[54, 55, 63, 64, 67]. One may therefore assume that the two exposures contribute more or less independently to risk.

Given this apparently relatively independent contribution to risk, apportionment by cause can be applied as a tradeoff between the contribution of asbestos exposure and the contribution of cigarette smoking to the degree of impairment, since COAD is manifested by and defined by increased resistance to airflow. A reasonable method is therefore needed for apportioning the relative contribution of cigarette smoking and asbestos in an asbestos-exposed worker who is impaired, with a reduced FEV_1. This might be done in three ways:

1. Assessing the rate of loss of pulmonary function characteristic of the worker, smoking or nonsmoking, prior to exposure to asbestos, extrapolating the rate of loss, and determining the difference between the predicted rate of loss and that observed, which is assumed to be due to asbestos exposure. The relative contribution of each to the last relevant set of pulmonary function studies would be the apportionment attributed to each cause.

   This approach is most rigorous but depends on having at least two FEV_1 determinations prior to beginning work involving exposure to asbestos. This is not realistic in most cases. Variability in spirometric measurements is enough to obscure or exaggerate such changes when the tests are performed in different laboratories. Workers who have had routine spirometry are also likely to have had the test as surveillance for dust exposure in an earlier job or because they had a lung disease; in either case the predictive value of the baseline rate of change of FEV_1 is reduced but it would be even more important to obtain individualized results. It may be challenged if the worker then quits smoking, although rates of decline in FEV_1 only recover after some time. Removal from exposure to asbestos would not normally present a problem in interpretation because the accelerated decline in FEV_1 continues for at least 10 years[55].
2. When a baseline FEV_1 is available, assume that the rate of loss of pulmonary function due to aging is the average of 30 ml/y, extrapolate the expected rate of loss to current pulmonary function, and determine the difference between the predicted rate of loss and that observed. This difference is assumed to be due to asbestos exposure. The relative contribution of each to the last relevant set of pulmonary function studies would be the apportionment attributed to each cause.

   This method can be used in cases where pre-exposure pulmonary function levels are not known, which is the

majority of cases. This is not individual-specific but it is based on group norms for rate of change of FEV_1. Spirometric variability remains a problem.

3. Assess current pulmonary function, and compare with predicted values, then apply a crude rule of thumb to the difference: 50% apportionment to asbestos and 50% to cigarette smoking, of the respiratory impairment. This method has the advantage of simplicity but cannot take into account degrees of exposure or smoking history. It is probably an overestimate (thereby "giving the benefit of doubt to the worker", appropriate to workers' compensation) since it is unlikely that asbestos exposure would be responsible for as much as 50% of isolated obstructive impairment.

Applying the criteria for substantial contribution, one may derive a reasonable test for substantial contribution in asbestos exposure, as demonstrated in the next section. As a practical matter, individual awards at such low levels of impairment in the absence of a test would be small but there could be many of them. A small error on the side of inclusiveness is not very expensive but the total absence of a test would place a huge demand on the system.

Substantial contribution in chronic obstructive airway disease

In chronic obstructive airways disease, the outcome is the physiological impairment. Apportionment of cause therefore apportions impairment, and vice versa. If more than half of the impairment is due to an occupational cause, then the disorder is presumptively occupational and qualifies as an occupational disease. If less than half, then the contribution may be significant but it is by definition not the major determinant of disease. If the impairment is not sufficient to push an otherwise fit person into a level of impairment recognized by workers' compensation, it would be inconsistent to call it a substantial contribution for purposes of compensation. Therefore an exposure that causes a lesion so trivial that it cannot be discerned in the contribution to total impairment cannot be considered a substantial contribution. As a practical matter, therefore, one is concerned about contributions to the apportionment of predominantly nonoccupational disease from, say, 5% to 50%.

Ohlson *et al.*[37] presented data that relate lung function as a percentage predicted from regression equations by exposure category for asbestos workers. These data are cross-sectional in a stable, aging workforce without evidence of asbestos-related disease or evidence of significant out-migration. Although a longitudinal study would be preferable, these data do reflect the realities of clinical presentation, as they would be enrolled as workers' compensation claims. Notwithstanding that the regression never dipped below the range of normal, their data provides a relationship between very mild impairment and exposure. These data are particularly useful in defining the relationship between exposure and response for changes so subtle that they could not be appreciated by any other means. The table is adapted in Table 1.

Table 1. Lung function as a percentage predicted from regression equations by exposure category for asbestos workers (Data from 37)

Fibre·years:	0 – 14 (n=41)	15 – 22 (n=42)	23 + (n=41)
FVC	96.1	95.4	94.6
FEV_1	92.8	91.8	90.5

There are two ways of reading the regression. It may be read as a prediction for the entire population and therefore a best estimate for the individual, or as an average for the population with variability among individual subjects, so that a small subset of subjects might have a markedly greater loss than the average. The authors comment that "the group exposed to dust with comparatively low asbestos fibre concentration had a minor impairment of lung function…", both smokers and nonsmokers, and variance was low in this population. They do not identify a subset with disproportionately poor pulmonary function, although such a subset would be of greatest concern.

The Ohlson data[37] show a linear relationship with a very slight slope and are clearly reflective of a mild effect in a population with generally preserved pulmonary function. It is therefore a useful data set for the purpose of defining substantial contribution. A longitudinal study would be even more useful.

The standard convention in pulmonary function testing is to consider both FVC and FEV_1 as abnormal only when they fall below 80% of predicted. Functional impairment for most people, other than athletes, is generally not demonstrable until at least this much function has been lost. This convention is reflected in the *AMA Guides to the Evaluation of Permanent Impairment*, which does not recognize impairment as existing until this threshold is reached. Category 1, involving either FVC or $FEV_1 > 80\%$ predicted, is associated with 0% impairment of the total person. FVC is less obviously linked to symptomatic impairment than FEV_1 and seems to be less impaired in asbestos-related disease than FEV_1, at least in the earliest stages. Therefore FEV_1 should be used as the most sensitive

indicator of effect. If one assumes that 20% of FEV_1 must be lost before impairment is obvious, what fraction of that 20% must result from a given cause before it can be considered "substantial"?

For a disorder to result in a loss of FEV_1 sufficient to push a normal person who smoked across the line into clinical impairment, perhaps half of this residual may be required; this is a clinical impression not easily validated by data. Thus, a level of exposure sufficient to result in loss of 5% of function is a reasonable threshold for what is substantial. This is also reasonable considering that it exceeds the measurement error of careful spirometry by the ATS criteria.

Referring to Table 1[37], a loss of only 5% of FEV_1 would correspond to approximately 10 fibre·years of asbestos exposure. This number can now be compared with other derivations as an estimate of a reasonable exposure level constituting substantial contribution.

If the effect of an exposure to asbestos, for example, was only to produce a pleural plaque, that might qualify as a tissue injury in pathological terms, but not as a cause of an outcome leading to impairment. The tissue injury did not interfere with function. In some compensation systems, the worker is still entitled to compensation for an asbestos-related condition, i.e. medical costs for annual surveillance, but not for permanent impairment. However, if one may demonstrate that the same exposure to asbestos resulted in a decrement in pulmonary function that falls outside the range of normal variability and could mean the difference between impairment and freedom from impairment in a worker developing chronic obstructive airways disease, that would constitute a substantial contribution. Unfortunately, there is no relationship demonstrable between the loading of fibres required to produce a plaque and that required to contribute to airflow obstruction, so plaques cannot be used as a marker of substantial contribution and the absence of plaques cannot be used to rule out a substantial contribution[6].

Conclusion

Asbestos-related diseases are attractive models for the application of apportionment. In practice, apportionment is less useful as a rigid approach or formula for managing claims than as a conceptual framework for thinking about the problem. The models presented here may serve as a general guide to the assessment of asbestos-related disease outcomes for purposes of compensation.

Asbestos-related outcomes vary greatly in their suitability for apportionment. For mesothelioma and asbestosis, apportionment is not a very meaningful process. For airflow obstruction, it is a complex and technical but theoretically valid approach. For lung cancer, it is complicated and there are no markers or approaches that support apportionment in the individual case. This means that different asbestos-exposed workers with different outcomes are being judged differently by the system of adjudication. In some cases, e.g. patients with asbestosis who have a predictably high cancer risk, the sequence of these outcomes are almost matters of chance and the injured worker may as easily presented with lung cancer first as asbestosis.

Unlike apportionment of impairment, where there are consensus standards such as the *AMA Guides to the Evaluation of Permanent Impairment*[68] apportionment by cause has achieved no consensus, defies the imposition of rigid standards, and is not convertible (as is percentage impairment of the total person) from one disease category to another. Within this class of injured workers, is it reasonable to apportion in some cases and not others simply because apportionment is possible in those cases?

This raises the issue of equity. On the one hand, it is standard operating procedure for the workers' compensation to evaluate hand injuries, occupational lung disease, noise-induced hearing loss, and brain injury by different criteria. The "apportioned" causation may be reflected in the apportioned impairment (in these cases always for aggravational injury) so that eventually these very different cases are evaluated on a comparable scale. However asbestos-related diseases reflect different outcomes of a common exposure in a situation where the effect is not aggravational but simultaneously causal. Is it reasonable to treat these related disorders so differently?

This is a fundamental issue in workers' compensation policy and falls outside the scope of this report. It is raised, however, to suggest that apportionment may not be equitable if its application is constrained in some cases more than others[4, 5].

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Industrial Health 2002, **40**, 313–319 **Original Article**

Influence of Frequency on Difference Thresholds for Magnitude of Vertical Sinusoidal Whole-Body Vibration

Yasunao MATSUMOTO[1]*, Setsuo MAEDA[2] and Yasushi OJI[1, 3]

[1] Department of Civil and Environmental Engineering, Saitama University, 255 Shimo-Ohkubo, Saitama, 338-8570, Japan

[2] Department of Human Engineering, National Institute of Industrial Health, 6-21-1 Nagao, Tama-Ku, Kawasaki, 214-8585, Japan

[3] Now at Obayashi Road Corporation, Japan

Received December 14, 2001 and accepted June 10, 2002

Abstract: Differences in vibration magnitude required for a human subject to differentiate whole-body vertical sinusoidal vibrations, difference thresholds for amplitude of sinusoidal vibration, have been determined at a vibration magnitude of 0.7 m/s^2 r.m.s. at five octave band center frequencies from 4 to 63 Hz and at 80 Hz. The median difference thresholds of 16 male subjects seated on a flat rigid seat were found between 0.037 and 0.046 m/s^2 r.m.s. at the frequencies used in this study. The subjects tended to be more sensitive to the change in vibration magnitude at 4 Hz than at 16, 31.5 and 63 Hz and less sensitive to the magnitude difference at 31.5 Hz than at 4, 8 and 80 Hz. The median relative difference thresholds, Weber's ratios, varied from 5.2% to 6.5% which were lower compared to the relative difference thresholds determined in the previous studies at frequencies where comparable data were available. The causes of the difference in the relative difference thresholds observed between this study and previous studies may include the difference in the psychophysical method used to determine the difference threshold.

Key words: Whole-body vibration, Human response, Difference threshold, Just noticeable difference, Magnitude, Frequency

Introduction

People are exposed to many kinds of whole-body vibration on a daily basis while involved in various activities for occupational, leisure, or other purposes. The exposures to whole-body vibration may cause adverse effects on occupants, ranging from uncomfortable feeling to injuries such as disorders of the spine[1)]. One of the differences between the occupational vibration exposures and other exposures is that occupants cannot easily avoid or control vibration exposures by themselves in occupational situation. In Japan, the Labour Standards Bureau Notification No. 547 reported that operations of various vehicles for a long period was included in five major causes of low back pain in workplaces[2)]. It can be assumed that low back pain in workers occurs when they are exposed to vibrations for a period longer than a certain period at magnitudes higher than a certain level. This does not mean that vibrations at low magnitudes which may not cause low back pain in workers are not required to address. Such vibrations can cause discomfort in workers or disturb their concentration on their work. It is therefore important for governments, employers or managers to reduce such vibration exposures as an improvement of occupational environment for health, including mental health, and productivity of workers.

When the reduction of the magnitude of vibration which causes adverse effects on people is sought, the knowledge about the amount of reduction in vibration magnitude that people can notice is useful information. In general, a minimum change in some aspect of stimulus that a human

*To whom correspondence should be addressed.

observer can detect is called by different terms: 'difference threshold', as used in this paper, 'difference limen (DL)' or 'just noticeable difference (JND)'. The difference thresholds for the vibration magnitude may be considered as a minimum target when the reduction of the vibration magnitude in workplaces is sought.

There have been some studies which investigates difference thresholds for magnitude of vibration. Two earlier studies have determined difference thresholds for random vibrations related to vibrations experienced in a car[3, 4]. Although these studies may have provided useful information about difference thresholds to car industries, understanding of the nature of difference thresholds for vibration magnitude, such as the effect of vibration frequency or the effect of vibration magnitude, may be improved more clearly in laboratory studies with more controlled input stimuli.

Two recent studies have investigated difference thresholds for amplitude of sinusoidal vibration with subjects seated on a flat rigid seat. Morioka and Griffin[5] have determined difference thresholds for vertical sinusoidal vibrations at two frequencies, 5 and 20 Hz, at two magnitudes, 0.1 and 0.5 m/s^2 r.m.s. The statistical analysis of the experimental data showed that the relative difference thresholds of about 10% did not differ significantly between the two vibration magnitudes or the two frequencies. It was stated that this finding was consistent with Weber's law: a change in a stimulus of any type that a person just notices is proportional to the size of the stimulus (i.e., the relative difference threshold obtained by dividing the difference threshold by the magnitude of the stimulus, $\Delta S/S$, or Weber's ratio, is constant for the stimulus). Difference thresholds for vertical sinusoidal vibration have also been determined by Bellmann *et al.*[6] at eight frequencies between 10 and 50 Hz at a vibration magnitude of 0.063 m/s^2. It was reported that the relative difference thresholds were about 1.5 dB (i.e. about 19%) and there was no statistically significant differences between the eight frequencies.

The perception thresholds of seated subjects exposed to vertical whole-body vibration determined in previous studies (e.g. Miwa[7], Parsons and Griffin[8], summarized in Griffin[1]) tended to be dependent on frequency: for example, lower thresholds at frequencies around 4 Hz. It might not be unreasonable to expect that the difference thresholds have a characteristic of frequency dependence similar to that of the perception thresholds, although the results of the two previous studies with sinusoidal vibration supported that the difference thresholds are independent of the vibration frequency.

The objective of the present study was to investigate the effect of vibration frequency on difference thresholds for amplitude of vertical sinusoidal vibration. A wider frequency range compared to those used in the previous studies was employed in the present study. Further understanding of the nature of the sensitivity of people to the change in magnitude of whole-body vibration was expected to be obtained in the present study so as to use better knowledge to improve occupational environment.

Experimental Method and Analysis

An experiment involving human subjects was conducted in the laboratory of the National Institute of Industrial Health, Kawasaki, Japan. An electro-magnetic shaker, Akashi AST-11V, with an power amplifier, Akashi E-DA, was employed in the experiment. It was specified by the manufacturer that the shaker system could be operated with a waveform distortion below 2% in the frequency range between 0.5 and 300 Hz. Sixteen healthy male volunteers aged between 21 and 23 yr took part in the experiment. The height and weight of the subjects were ranged from 1.64 to 1.81 m and from 49 to 77 kg, respectively. A subject was seated on the top face of the shaker which was flat and had a circular shape with a diameter of 0.4 m. No backrest was provided during the experiment. The feet of the subject was supported by a stable footrest whose height was 0.39 m below the top of the shaker. The subject wore ear defenders so as to prevent from perceiving vibration by accompanied noise generated by the shaker. A vertical, or z-axis[9], acceleration at the interface between the top face of the shaker and the subject was measured with a piezo-electric accelerometer, B&K 4322, with a charge amplifier, B&K 2635.

Difference thresholds for the perception of the change in the vibration magnitude, ΔS, was determined with vertical sinusoidal vibrations at six different frequencies: 4, 8, 16, 31.5, 63 and 80 Hz. For the vibrations at each frequency, a subject was exposed to pairs of sinusoidal vibrations. A pair of vibrations consisted of a reference vibration at a nominal magnitude of 0.7 m/s^2 r.m.s. and a test vibration. The duration of each vibration was 4 seconds and the interval between the two vibrations was 2 seconds. For each vibration stimulus, the waveforms for the first and last 0.5 seconds were tapered so as to eliminate a transient response of the shaker that had an effect on the perception of input stimulus of the subject in a preliminary experiment conducted prior to the main experiment. At each trial, the subject was asked to judge the difference in the vibration magnitude between the reference vibration and the test vibration by using the following three wordings: 'the first vibration was greater',

Table 1. Four types of presentation of stimuli used in the experiment

	Test vibration magnitude greater than reference	Test vibration magnitude lower than reference
Reference → Test	Type I	Type II
Test → Reference	Type III	Type IV

'the second vibration was greater', or 'I did not perceive a difference between the two stimuli'.

The magnitude of the test vibration was either greater or lower than the magnitude of the reference vibration by 0.25 dB, about 2.9%, at the first trial. The magnitude difference between the two stimuli increased with 0.25 dB increment steps by changing the magnitude of the test vibration (i.e. either increasing or decreasing the magnitude of the test vibration, depending on the magnitude of the test vibration at the first trial) until the subject perceived the magnitude difference correctly. It was decided to terminate a series of trials if the magnitude difference reached 3.0 dB, although this rule did not apply to any series in this experiment. The difference threshold for a series of trials, ΔS_i, was then determined by calculating an arithmetic average of the magnitude difference at the last trial when the subject perceived the magnitude difference correctly and the magnitude difference at the second trial from the last trial. The vibration magnitude measured with an accelerometer was used to calculate the differences in magnitude between the reference vibration and the test vibration. The relative difference threshold for a series of trials was calculated by dividing the difference threshold obtained for the series of trials, ΔS_i, by the magnitude of the reference vibration, S (i.e. a relative difference threshold for a series = $\Delta S_i/S$).

The order of the reference vibration and the test vibration in a pair was changed for each series of trials so as to reduce the influence of the order of vibrations on the difference thresholds. Table 1 shows the four types of presentation of stimuli used in the experiment: whether the magnitude test vibration increased or decreased, and whether the reference vibration was presented first or second. The subject was presented stimuli in all four types shown in Table 1 in a randomized order for each frequency of vibration. A series of the four presentation types was repeated three times so that the difference thresholds for each presentation type were obtained by taking the average of three repetitions. The order of vibration frequencies presented was different for each subject. The subject completed the experiment with 72 series of trials (i.e. four series of trials with three repetitions for six frequencies), which took about one and a half hours, including a short break. The subject was informed when the frequency of vibration tested was changed, although any other methods of presentation of vibration were not known by the subject.

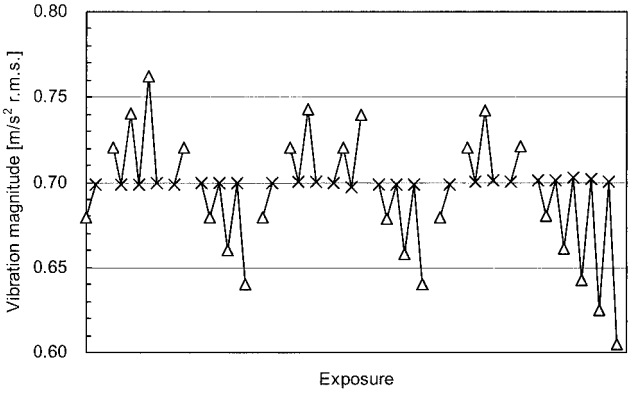

Fig. 1. Example of the history of vibration exposures measured for a subject to determine the difference thresholds for sinusoidal vibration at a frequency.
The history of exposures was represented by the magnitude of reference and test vibrations. ×: reference vibration; △: test vibration. A line indicates a series of trials with a type of presentation of stimuli. The order of the presentation types shown in Table 1 was Type IV → Type III → Type I → Type II, which was repeated three times.

Results

Example of exposure history to determine difference threshold

Figure 1 shows an example of the history of vibration exposures recorded for a subject from which the difference threshold for sinusoidal vibration at a frequency was determined. The history of exposures was represented by the magnitude of reference and test vibrations to which the subject was exposed. In this example, the order of the four presentation types shown in Table 1 was Type IV, Type III, Type I, and Type II, which was repeated three times. The difference threshold obtained with the data shown in Fig. 1 was close to the median difference threshold of the 16 subjects.

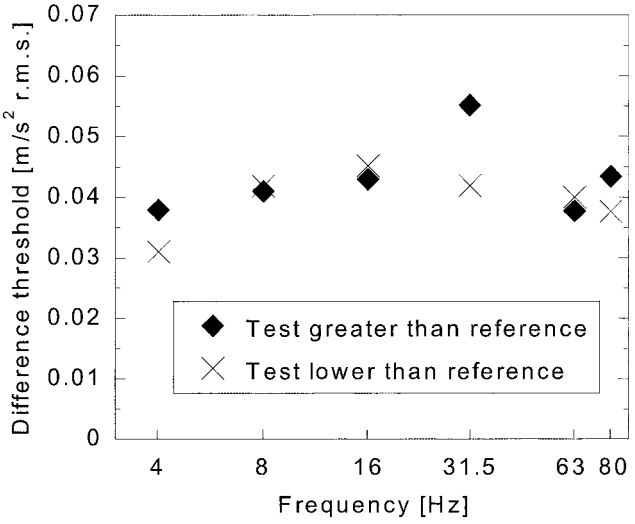

Fig. 2. Median difference thresholds determined with test vibrations at magnitudes greater than the magnitude of the reference vibrations, 0.7 m/s^2 r.m.s., (i.e. Types I and III in Table 1) and with test vibrations at magnitudes lower than the magnitude of the reference vibrations (i.e. Types II and IV).

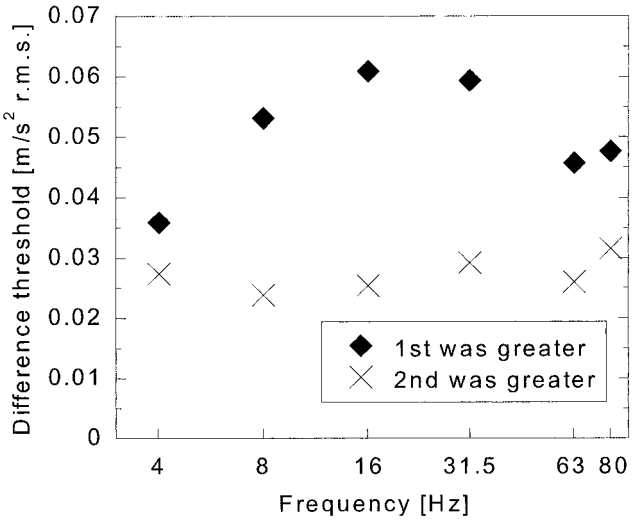

Fig. 3. Median difference thresholds obtained when the magnitude of the vibration presented first was greater than the magnitude of the vibration presented second (i.e. Types II and III in Table 1) and when the magnitude of the vibration presented second was greater than the magnitude of the vibration presented first (i.e. Types I and IV).

Influence of magnitude of test vibration on difference threshold

The median difference thresholds of the 16 subjects determined with the test vibrations at magnitudes greater than the magnitude of the reference vibrations, 0.7 m/s^2 r.m.s., (i.e. Types I and III in Table 1) and with test vibrations at magnitudes lower than 0.7 m/s^2 r.m.s. (i.e. Types II and IV) were compared in Fig. 2. The difference threshold determined with test vibrations greater than 0.7 m/s^2 r.m.s. tended to be greater than the difference threshold determined with test vibrations lower than 0.7 m/s^2 r.m.s. at 31.5 Hz as observed in Fig. 2. However, there was no statistically significant difference in the difference thresholds caused by the difference in the magnitude of test vibration with the Wilcoxon matched-pairs signed ranks test ($p>0.05$). The difference thresholds determined in the two conditions have, therefore, been combined in the analysis presented later in this paper.

Influence of order and relative magnitude of two vibrations

It is generally known that when the magnitudes of two stimuli of any type in a series are compared the magnitude of the stimulus presented second tends to be judged relatively greater than the magnitude of the stimulus presented first (i.e. negative time error[10]). This order effect was observed in the results of this experiment. Figure 3 compares the median difference thresholds of the 16 subjects obtained when the magnitude of the vibration presented first was greater than the magnitude of the second vibration (i.e. Types II and III in Table 1) and when the magnitude of the vibration presented second was greater than the magnitude of the first vibration (i.e. Types I and IV). It was clear that the difference thresholds determined when the first vibration was greater than the second was greater than the difference thresholds determined when the second vibration was greater than the first. This indicates that the subjects might judge the magnitude of the second vibration relatively greater than the magnitude of the first vibration. The differences between the difference thresholds determined in the two conditions were statistically significant at all frequencies used in this experiment (Wilcoxon, $p<0.05$ at 4, 63 and 80 Hz, $p<0.01$ at 8, 16 and 31.5 Hz). The median difference thresholds determined when the second vibration was greater than the first vibration varied 0.025 to 0.032 m/s^2 r.m.s. (i.e., 0.3 to 0.4 dB) as shown in Fig. 3. An increment step for vibration magnitude smaller than 0.25 dB may be required to determine the difference threshold more accurately, although it was difficult to produce a smaller increment step than 0.25 dB accurately due to the limitation of the shaker system.

Influence of frequency

The difference thresholds obtained with all the presentation types shown in Table 1 were averaged to determine the difference thresholds at each frequency used for each subject.

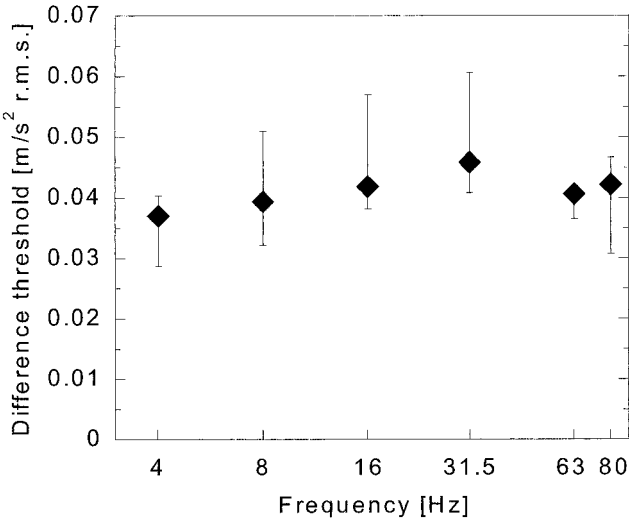

Fig. 4. Medians and inter-quartile ranges of the difference thresholds of the 16 subjects determined by combining the difference threshold obtained with the four presentation types shown in Table 1.

It was intended that the combination of the difference thresholds obtained with the four presentation types reduced the effect of the order of vibration presentation on the difference thresholds mentioned in the preceded section. The medians and inter-quartile ranges of the difference thresholds of the 16 subjects are presented in Fig. 4. The difference thresholds determined for different frequencies by averaging the difference thresholds obtained with the four presentation types are compared by using the Wilcoxon matched-pairs signed ranks test in Table 2. These results show that the difference thresholds at 4 Hz tended to be lower than the difference thresholds at the other frequencies (significantly lower than those at 16, 31.5 and 63 Hz (Wilcoxon, $p<0.05$)). The difference thresholds at 31.5 Hz tended to be higher compared to the difference thresholds at the other frequencies (significantly higher than those at 4, 8 and 80 Hz (Wilcoxon, $p<0.05$)).

Discussion

The difference thresholds determined in this study showed that the sensitivity of the subjects to the change in vibration magnitude around the reference vibration magnitude used tended to be dependent on frequency: higher sensitivity at frequencies of 4 and 8 Hz, particularly at 4 Hz, than at frequencies around 31.5 Hz. The perception thresholds of seated subjects exposed to whole-body vibration measured in previous studies (e.g. Miwa[7], Parsons and Griffin[8]) showed a similar trend to the frequency dependence observed in the difference thresholds measured in this study: lower perception thresholds in the frequency range around 4 Hz. This may be consistent with a hypothesis that a subject tend to be sensitive to the change in vibration magnitude at frequencies where he/she can perceive vibration at lower magnitude.

In the previous studies of the difference thresholds by Morioka and Griffin[5] and Bellmann *et al.*[6], it was concluded that there was no frequency dependence of difference thresholds found. It was reported by Morioka and Griffin[5] that there was no statistically significant difference in the difference thresholds between the two vibration frequencies at the two vibration magnitudes used. Although there was a statistically significant difference between the difference thresholds obtained at frequencies of 4 and 16 Hz in this study, the frequencies used in Morioka and Griffin[5], 5 and 20 Hz, were not used in this study so that discussion based

Table 2. Results of Wilcoxon matched-pairs signed ranks test for comparison of difference thresholds determined for 6 frequencies with the 16 subjects (*$p<0.05$, **$p<0.01$). (16>4), for example, indicates that the difference threshold at 16 Hz appeared to be statistically significantly greater than the difference threshold at 4 Hz

	4 Hz	8 Hz	16 Hz	31.5 Hz	63 Hz	80 Hz
4 Hz	–	0.079	0.011* (16>4)	0.004** (31.5>4)	0.039* (63>4)	0.148
8 Hz		–	0.223	0.026* (31.5>8)	0.756	0.959
16 Hz			–	0.609	0.211	0.211
31.5 Hz				–	0.098	0.039* (31.5 > 80)
63 Hz					–	1.000
80 Hz						–

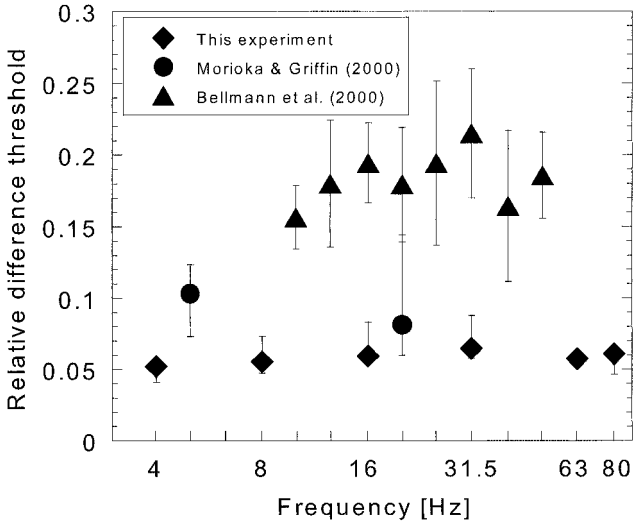

Fig. 5. Comparison of the relative difference thresholds determined in this study and in previous studies.

The medians and inter-quartile ranges of the 16 subjects determined at a vibration magnitude of 0.7 m/s^2 r.m.s. for this study. The medians and inter-quartile ranges of 12 subjects determined at 0.5 m/s^2 r.m.s. for Morioka and Griffin[5]. The means and standard deviations of 8 subjects determined at 0.063 m/s2 for Bellmann *et al.*[6].

on a direct comparison between the results from the two studies may not be reasonable. The data reported by Bellmann *et al.*[6] show that the mean difference threshold was greatest at a frequency of 31.5 Hz. This trend is consistent with the results obtained in this study. It was reported by Bellmann *et al.*[6] that there was no significant difference between the difference thresholds measured at eight frequencies investigated at $p<0.01$, a lower level than $p<0.05$ used in this study. The frequencies for which the difference threshold was significantly different from the difference threshold measured at 31.5 Hz in this study were 4, 8 and 80 Hz that were not covered by the frequency range used by Bellmann *et al.*[6]. Therefore, with respect to the influence of vibration frequency, although the results of this study implies that the difference threshold is dependent on frequency while Bellmann *et al.*[6] reported no statistically significant difference between the difference thresholds at the eight frequencies investigated, the results obtained in this study might not be inconsistent with the data reported by Bellmann *et al.*[6].

The relative difference thresholds determined by combining the relative difference thresholds obtained with the four presentation types in this study are compared with the relative difference thresholds measured by Morioka and Griffin[5] and Bellmann *et al.*[6] in Fig. 5. The data presented for Morioka and Griffin[5] in Fig. 5 are the medians and inter-quartile ranges of the difference thresholds for 12 male seated subjects measured with sinusoidal vibrations at two frequencies, 5 and 20 Hz, at a magnitude of 0.5 m/s^2 r.m.s. They also measured the difference thresholds at a vibration magnitude of 0.1 m/s^2 r.m.s., which are not presented in Fig. 5. The results presented for Bellmann *et al.*[6] are the means and standard deviations of the difference thresholds of two female and six male seated subjects determined with sinusoidal vibrations at eight third-octave band center frequencies from 10 to 50 Hz with a reference vibration magnitude of 0.063 m/s^2.

If the difference threshold for the magnitude of whole-body vibration followed Weber's law, the difference threshold obtained at different vibration magnitudes would be constant. Morioka and Griffin[5] concluded that their results were approximately consistent with Weber's law, although there was a trend for the relative difference thresholds to reduce with increasing vibration magnitude. However, if Weber's law holds for the magnitude of whole-body vibration, the results from the three different studies shown in Fig. 5 should not be expected to be constant, partly because the psychophysical method used to determine the difference threshold in the three studies were different. The method used in this study may be categorized as the 'method of limits': if the response that a subject can detect the difference in the magnitude between the reference vibration and the test vibration is defined as the 'positive' response and this response follows a typical psychometric function, the method used in this study estimates difference thresholds at the probability of the positive response of 50%[11]. The two previous studies mentioned above used the up-and-down transformed response (UDTR) methods: Morioka and Griffin[5] used the method that estimates difference thresholds at 79.4% positive response and Bellmann *et al.*[6] used the method that estimates difference thresholds at 70.7% positive response. Maeda and Griffin found that vibrotactile thresholds on the finger were influenced by psychometric methods that estimate positive responses at different probabilities[12]. Other factors, including the posture of subjects, the condition of footrest and backrest, the increment size for vibration magnitude, used in this study was similar to those used in Morioka and Griffin[5], but different from those in Bellmann *et al.*[6]. Therefore, the difference in the psychophysical method may have contributed to lower relative difference thresholds obtained in this study compared to those obtained in Morioka and Griffin[5], while the causes of the differences in the relative thresholds observed between this study and Bellmann *et al.*[6] could include various other factors. The details of experimental conditions and

procedures were not given fully by Bellmann *et al.*[6] so that it was difficult to identify the causes of the differences between their experimental results and the results obtained in this study. The influence of the psychophysical method on the difference thresholds may be required to be investigated in future study.

Conclusions

For vertical sinusoidal vibrations at octave band center frequencies from 4 to 63 Hz and at 80 Hz, difference thresholds for vibration magnitude have been measured with 16 male subjects. The median difference thresholds ranged from 0.037 to 0.046 with a reference vibration magnitude of 0.7 m/s^2 r.m.s. The difference thresholds may be dependent on frequency as the perception thresholds for whole-body vibration are: it was found that the difference threshold determined at 4 Hz was lower than the difference thresholds at 16, 31.5 and 63 Hz and the difference threshold determined at 31.5 Hz was greater than those at 4, 8 and 80 Hz.

The median relative difference thresholds, Weber's ratios, of the 16 subjects were found to vary from 5.2% to 6.5%. The relative difference thresholds obtained in this study tended to be lower than the relative difference thresholds reported in available previous studies at frequencies where the comparison was possible. The effect of the psychophysical method to determine the difference threshold may be one of the causes of the difference observed between this study and previous studies and needs to be investigated in further study.

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Industrial Health 2002, **40**, 320–327 **Original Article**

Effects of Electromagnetic Radiation (Bright Light, Extremely Low-Frequency Magnetic Fields, Infrared Radiation) on the Circadian Rhythm of Melatonin Synthesis, Rectal Temperature, and Heart Rate

Barbara GRIEFAHN[1]*, Christa KÜNEMUND[1], Meinolf BLASZKEWICZ[1], Alexander LERCHL[2] and Gisela H. DEGEN[1]

[1] Institute for Occupational Physiology at the University of Dortmund, Ardeystr. 67, D-44139 Dortmund, Fed. Rep. Germany

[2] International University Bremen, Campus Ring 1, D-28758 Bremen, Fed. Rep. Germany

Received October 23, 2001 and accepted June 24, 2002

Abstract: Electromagnetic spectra reduce melatonin production and delay the nadirs of rectal temperature and heart rate. Seven healthy men (16–22 yrs) completed 4 permuted sessions. The control session consisted of a 24-hours bedrest at < 30 lux, 18°C, and < 50 dBA. In the experimental sessions, either light (1 500 lux), magnetic field (16.7 Hz, 0.2 mT), or infrared radiation (65°C) was applied from 5 pm to 1 am. Salivary melatonin level was determined hourly, rectal temperature and heart rate were continuously recorded. Melatonin synthesis was completely suppressed by light but resumed thereafter. The nadirs of rectal temperature and heart rate were delayed. The magnetic field had no effect. Infrared radiation elevated rectal temperature and heart rate. Only bright light affected the circadian rhythms of melatonin synthesis, rectal temperature, and heart rate, however, differently thus causing a dissociation, which might enhance the adverse effects of shiftwork in the long run.

Keywords: Bright light, Extremely low-frequency magnetic field, Infrared radiation, Melatonin, Rectal temperature, Heart rate

Introduction and Objectives

The synthesis of melatonin, the main secretory product of the pineal gland, is known to follow an endogenous circadian rhythm that is controlled by the central circadian pacemaker in the suprachiasmatic nuclei (SCN) of the anterior hypothalamic area and entrained by the light-dark cycle to a 24-hours rhythm. The information about light is transmitted from the retina to the pacemaker and then via several neurons to the pineal gland, there inhibiting melatonin synthesis, which is therefore significantly elevated only during dark. Melatonin in turn possibly mediates the entrainment of the diurnal alterations of physiological functions (core body temperature, heart rate etc) by acting on the SCN which contain high amounts of melatonin receptors[1]: The Mel1a melatonin receptor gene is expressed in human suprachiasmatic nuclei.

Apart from natural light, melatonin production is reliably suppressed by artificial light where the extent of the effects depends on intensity, wavelength, duration, and timing[2]. The period of the usual onset and rise of nocturnally elevated melatonin synthesis seems to be particularly sensitive not only to light but also to the impact of magnetic fields[3, 4].

Similar, but far smaller effects were evoked in animal experiments by other parts of the electromagnetic spectrum[5], namely by UV-A radiation[6] and extremely low-frequency magnetic fields[7]. These latter effects are debated for human beings[5]. A few studies executed so far revealed inconsistent

*To whom correspondence should be addressed.

and even contradictory results[8–11]. The effects of infrared radiation were not yet studied in human beings.

The present paper concerns the hypothesis that different parts of the electromagnetic spectrum, i.e. a) moderate bright light, b) a very strong magnetic field, and c) infrared radiation suppress (at least partially) melatonin synthesis. As exposure is ceased before the usual termination of melatonin production[12, 13] suppression is followed by a rebound. This delayed and excessive though shortened melatonin synthesis might be the earliest indicator of an effect on the circadian rhythm. These exposures are wide spread and concern e.g. electricians and railway workers (extremely low frequency magnetic fields), precision engineers (bright light) and steel workers (longwave radiation) and the effects studied here are expected during nightshifts. As melatonin concentrations are closely and inversely coupled with core temperature and thereby with heart rate, corresponding though small effects on both the latter variables were expected[14, 15].

Material and Methods

Participants

The study was approved by the local Ethics Committee. Eight healthy young men were selected using a questionnaire on health, particularly on symptoms and diseases concerning the central nervous system. They were informed about the goal and the procedure of the study and gave their written consent. A participant withdrew just before his second session. The data of the remaining 7 men are listed in Table 1.

Table 1. Means, standard deviations, minima and maxima of the 7 male participants

	mean ± sd	min – max
Age [years]	19.0 ± 2.2	16 – 22
Weight [kg]	79.7 ± 20.6	57.5 – 119.7
Height [cm]	183.6 ± 6.6	176 – 197
BMI	23.4 ± 4.6	18.6 – 30.8

Experimental design

Each subject participated at weekly intervals in 4 systematically permuted 24-h sessions, a control session, which was performed as a 'constant routine' (see Experimental procedure), and 3 experimental sessions which differed from that protocol during an 8-h period, where a physical stress was applied from 5 pm to 1 am, i.e. bright light (1 500 lux), a continuous magnetic field (16.7 Hz as emitted by railways, 0.2 mT), or infrared radiation (1.5–6.5 μm, 65°C) as shown in Table 2. All experiments were performed during summer time (May–July). The clock-time was converted into Central European Time (CET, actual summer time was 1 hour later).

Technical equipment

The experiments were performed in 3 rooms, which were particularly designed and equipped for the respective purpose. The control session and exposure to the magnetic field were performed in the same room. So, presupposing that the participants are unable to perceive the field consciously, they were blind concerning the date and the time of field exposure. This was of course impossible for infrared radiation and exposure to light, to which the participants became aware at their onsets.

Bright light (BL): Exposure to bright light was performed in a chamber where the entire ceiling was covered with fluorescent separately controlled tubes that provided a white light (OSRAM L58W/12, Lumilux de Luxe, daylight, 12–950). Flickering was avoided by a special device (Professional QTP 2*58/230-240).

Magnetic field (MF): Two Helmholtz-coils with a diameter of 1.80 m each, were located with a distance of 92 cm to both sides of the bed which contained no metals. The hum from the coils was not audible. Homogenity of the horizontally oriented field was assessed with measurements at 14 grid points in up-down direction and 12 points each in back-front and in left-right direction. The results revealed a minor decrease of the intensity at the margins of the field (up-down (mean/sd): 0.171/0.030 mT; back-front: 0.177/0.022 mT; left-right: 0.217/0.011 mT). The coils were not

Table 2. Experimental conditions during 4 sessions

	Physical stress (5 pm–1 am)		
	Magnetic field	Bright light	Infrared radiation
Control	–	< 30 lux	18°C
Magnetic field	16.7 Hz, 0.2 mT	< 30 lux	18°C
Bright light	–	1 500 lux	18°C
Thermal radiation	–	< 30 lux	t_r: 65°C, t_a: 18°C

energized during the control session.

As humans need much higher illumination levels than animals for melatonin suppression, the same might be true for magnetic fields. Thus, the flux density was set to a level that occurs only occasionally at the workplaces of engine drivers.

Infrared radiation (IR): Exposure to infrared radiation took place in a climatic chamber that has the same specifications as the 'bright light chamber', where radiation temperature (t_r), air temperature (t_a), humidity (RH) and air velocity (v_a), can be varied in large ranges and precisely adjusted (t_a: ± 0.1°C, RH: ± 0.5%, v_a: ± 0.01 m/s).

Experimental procedure

Prior to each session the participants assessed their actual health state and stated whether they had consumed alcohol or drugs. Based on the answers none of the experiments was postponed. After the application of the thermistors for rectal temperature and the electrodes for the electrocardiogram the participants went to bed. The subjects did not receive instructions concerning a strict time schedule prior to the sessions.

The control sessions were performed as 'constant routines', which consisted of a strict bedrest for 24 hours (from 11 am to 11 am) while the environmental conditions were strictly controlled; air temperature was 18°C, illumination < 30 lux and sound pressure level < 50 dBA. The participants were not allowed to sit or to leave the bed except for the lavatory. After saliva sampling they received snacks (200–400 kJ) and water or herb tea ad libitum. The remains were removed half an hour later to avoid effects on the next sampling. So, the experimenters entered the test rooms every 30 minutes. Apart from the physical stress from 5 pm to 1 am the procedure was the same during exposure sessions.

The participants were advised to read between 5 pm and 1 am in each session. At any other time they could read or close their eyes. Apart from hourly saliva sampling, they were not prevented from sleep. They were observed by means of a video camera and could communicate with the experimenter via an intercom system at any time.

Melatonin concentration: Saliva samples were collected at hourly intervals using the Salivette™ (Sarstedt, Nuembrecht, FRG), a cotton wool swab which was moved within the mouth until soaked with saliva. The swabs were immediately thereafter centrifuged and the saliva was then stored at –20°C until assayed. Melatonin concentrations were determined by means of a commercial competitive radioimmunoassay (RIA, IBL, Hamburg, FRG) with ^{125}I-labelled melatonin which is registered with γ-counting and finally analyzed with a standard curve. The limit of detection is about 0.8 pg/ml saliva. Most samples were analyzed in duplicate. The within and between variability was 11.5% and 2.6% as compared to a reference value of 11.1 pg/ml.

Rectal temperature and heart rate

Rectal temperature was measured continuously with thermistors (YSI 401 Yellow Springs), 10 cm beyond the sphincter; heart rate was calculated from the continuously recorded ECG (the leads were not disturbed by the magnetic field) and both variables were averaged and stored each consecutive minute.

Statistics

The temporal parameters of melatonin synthesis (onset, acrophase, offset), of rectal temperature and heart rate (acrophase, nadir) were derived from fitted curves. Two models were applied to the courses of melatonin synthesis. The model proposed by Brown *et al.*[16] was used to estimate the individual onset and offset. The acrophase was estimated from the model provided by Lerchl and Partsch[17]. The correlation between observed and predicted melatonin levels was sufficiently high and R-square was below 0.8 in only 1 case and in 86% above 0.9.

The analysis of rectal temperature and heart rate rhythms was based on the harmonic-regression-plus-correlated-noise model proposed by Brown and Czeisler[18], which was adjusted insofar as the periodic signal was described by a 3 harmonic-regression model and the correlated-noise in the data was assumed to be a first-order autoregressive process. The model parameters were estimated by a maximum likelihood procedure. The fitted curves correlated very well with the recorded data. The coefficient (R-square) was always > 0.9.

As rectal temperature declined initially (due to physical inactivity) in all test persons, values for the first 2 hours were excluded from the overall assessment. The remaining data were then averaged over every consecutive 10-min interval. For the parameter estimation in the harmonic-regression model the circadian period was assumed to be constant at 24 hours and the nadirs were computed numerically by a Newton-Raphson algorithm.

The differences between the control situation and the exposure sessions and the corresponding p-values were calculated with the Wilcoxon test (2-tailed).

Results

The courses of melatonin levels and of rectal temperature

Figures 1 to 3 present salivary melatonin levels, rectal

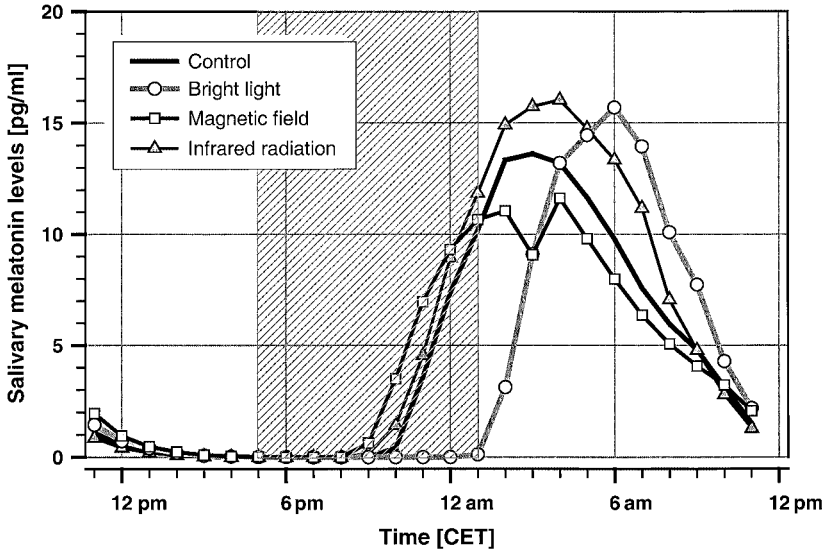

Fig. 1. Means of estimated hourly determined salivary melatonin concentrations during 4 sessions (control, bright light, magnetic field, infrared radiation) in 7 healthy young men.

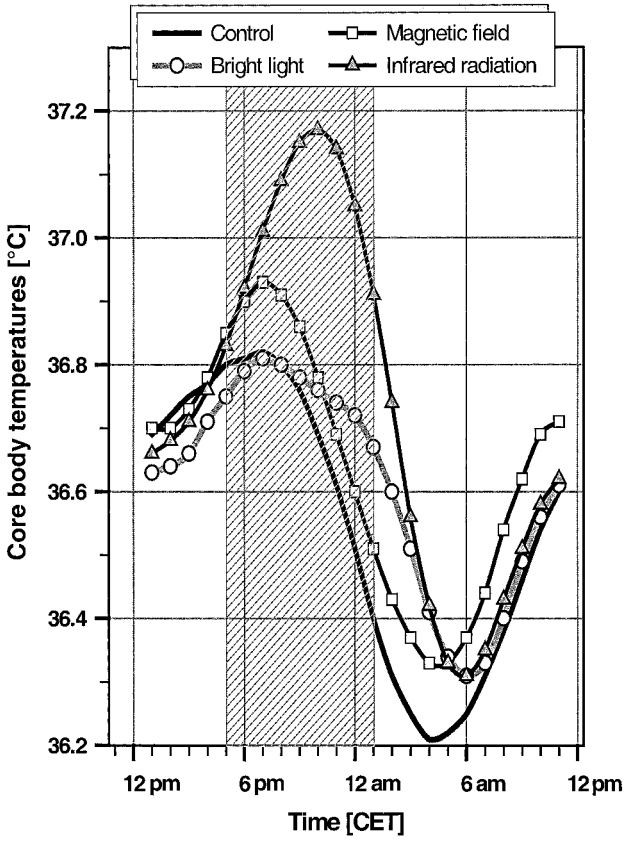

Fig. 2. Means of estimated (core body) rectal temperature during 4 sessions (control, bright light, magnetic field, infrared radiation) in 7 healthy young men.

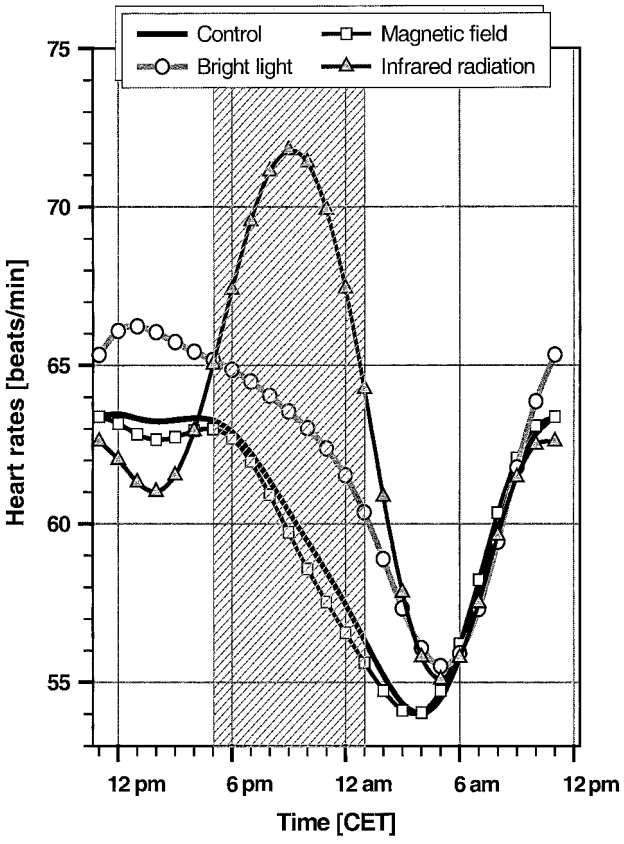

Fig. 3. Means of estimated heat rate during 4 sessions (control, bright light, magnetic field, infrared radiation) in 7 healthy young men.

Table 3. Temporal and quantitative parameters of salivary melatonin concentrations, rectal temperature and heart rate, medians, Wilcoxon signed rank test

parameter	Differences between control condition and physical stress					
	Bright light-control		Magnetic field-control		Infrared radiation-control	
	mean diff.	p-value	mean diff.	p-value	mean diff.	p-value
melatonin synthesis: temporal parameters (real times/durations)						
onset [h:min]	2:53	0.016	–0:46	0.688	–0:02	0.469
acrophase [h:min]	2:04	0.031	0:22	0.688	0:30	0.813
offset [h:min]	1:04	0.297	0:53	0.688	0:31	1.000
duration [h:min]	–1:44	0.031	0:12	0.813	0:30	0.813
core body temperature						
amplitude [°C]	–0.01	0.530	0.02	1.000	0.10	0.026
nadir [h:min]	1:38	0.026	0:13	1.000	0:49	0.053
delay to melatonin acrophase [h:min]	–0:42	0.813	–0:19	0.938	1:33	0.016
heart rate						
amplitude [bpm]	1.3	0.688	0.8	0.578	3.7	0.016
nadir [h:min]	1:12	0.031	–0:16	0.688	0:40	0.031
delay to melatonin acrophase [h:min]	–1:13	0.563	–0:38	0.688	1:07	0.078

temperature, and heart rate as averaged over the fitted curves, separately for the 4 conditions. The differences between the control situation and the exposure sessions and the corresponding p-values are listed in Table 3 for onsets, acrophases, and offsets of melatonin synthesis, for the nadirs of rectal temperature and heart rate and their delays to melatonin acrophase.

Bright light: Melatonin synthesis was completely suppressed during light exposure in each participant but immediately resumed upon return to dim light and increased then steeply to slightly higher maxima than during the control condition. Despite the significant delays of onsets and acrophases (2h53', p=0.016; 2h04', p=0.031), melatonin production ceased at almost the same time as during the control, thus reducing significantly the duration (1h44', p=0.031) and thereby total production of melatonin (as indicated by the area under the curve, 24 pg $ml^{-1}hrs^{-1}$, p=0.047).

The decrease of rectal temperature was delayed and the nadir occurred 1h38' later than during the control (p=0.026). The nadir of heart rate was delayed by 1h12' (p=0.031).

Magnetic field: Though the averaged melatonin curve indicates a slightly reduced amplitude, the magnetic field had no effects, neither on the temporal nor on the quantitative parameters of salivary melatonin, rectal temperature, and heart rate.

Infrared radiation: None of the melatonin parameters were significantly different from the control condition. Both rectal temperature and heart rate increased significantly during exposure to infrared radiation and the amplidudes were significantly higher than during control, i.e. by 0.10°C (p=0.026) and 3.7 beats per minute (p=0.016), respectively. The nadirs were delayed by 49 min and 40 min, respectively (p=0.053, p=0.031). The temporal distance of the nadirs to melatonin acrophase increased significantly for rectal temperature by 1h33' (p=0.016) and the increased delay for heart rate reached borderline significance (1h07', p=0.078).

Discussion

Methodological aspects

Salivary melatonin levels: Melatonin synthesis was indicated by hourly determined salivary concentrations. This is justified by the high correlation with plasma levels as reported e.g. by Deacon and Arendt[19], and Kennaway and Voultsios[20] and is advantageous as blood sampling is less acceptable for many persons.

Experimental protocol: The monitoring of melatonin and of rectal temperature determined the experimental protocol. Concerning the highly reproducible individual diurnal pattern of melatonin synthesis on the one hand and the large inter-individual differences on the other hand[21, 22], within-subject comparisons between control conditions and exposure sessions are deemed mandatory.

Core body temperature and heart rate are easily affected by many influences and the constant routine protocol was developed to minimize possible masking effects[23]. This protocol was considered as appropriate not only for the

control session but also for the detection of even small effects as expected e.g. by magnetic fields. As sleep does not affect melatonin synthesis[24] the participants were not prevented from sleep and a possible effect on rectal temperature is considered to be minimal as the experimenters entered the rooms at least every 30 minutes (see Materials and Methods), thus allowing only short sleep periods.

Influences on melatonin synthesis: Melatonin levels are almost inert against environmental and behavioral influences but affected by posture where plasma and salivary levels decrease in the supine and raise in the erect posture[19]. As constant levels are reached after about 20 minutes, the participants remained supine throughout the 24 hours period and left the bed only for the lavatory soon after saliva sampling.

Physical stress

Bright light: An exactly equal illumination for each participant requires rigorous experimental procedures as e.g. applied by Brainard *et al.*[2] who dilated the pupils and used an ophthalmological head holder to direct the gaze towards the light source. This is unacceptable for long-term exposures and the participants of the present study were allowed to turn their heads which caused illumination levels that varied from 1 500 to 2 500 lux for the gaze directed towards the walls and the light source, respectively. This rather low illumination suppressed melatonin synthesis nevertheless completely in any of the 7 subjects. This is explained by the fact that the period between the onset and the acrophase of melatonin synthesis is particularly sensitive against melatonin suppressive stress[3, 4].

Exposure in the present study was terminated well before the (usual) offset of melatonin synthesis, thus allowing rebound effects, which are differently understood in the literature. Here, this term is defined as any post-exposure excession (i.e. a shift) as compared to the corresponding time during the control session.

Melatonin synthesis was immediately resumed after return to dim light (Fig. 1) and the significantly delayed acrophase indicates a true rebound. This is supported by Beck-Friis[12] who determined rebounds in 3 persons whom they exposed to bright light in the early night (2 500 lux, 10 pm–11 pm) and by Horne *et al.*[13] who reported that urinary excretion of 6-hydroxymelatonin sulfate (6-OHMS) decreased during nocturnal exposure to bright light and increased thereafter. As indicated by the shift of the melatonin acrophase and of the nadirs of rectal temperature and heart rate as well as by their stable relation to each other, it is justified to assume a shift of the circadian system. The latter is usually verified with a post-treatment constant routine (e.g. after night shifts). It is, however, conceivable, that the immediate light-induced delay of melatonin acrophase predicts the consecutive shift of the circadian phase.

The delay of melatonin production was almost twice or thrice as long as the delays of the nadirs of rectal temperature and heart rate, respectively, thus indicating an internal dissociation of physiological rhythms. Internal dissociations occur typically during shiftwork and it might be speculated whether bright light enhances this effect and consequently accelerates the development of medical symptoms which are expected in the long run in shiftworkers.

Magnetic fields: None of the parameters derived from the 3 physiological variables revealed alterations that can be related to the impact of the continuously applied magnetic field. This is in accordance with the studies of Åkerstedt *et al.*[25] and Selmaoui *et al.*[10] whose subjects slept under the influence of a continuous though much weaker magnetic field (50 Hz, 1 μT and 10 μT, respectively). As the organism usually reacts more to changes than to even stress, Graham *et al.*[8, 9] and Wood *et al.*[4] exposed their subjects to an intermittent magnetic field with on-/off-periods of 15 seconds. Graham *et al.*[8, 9] reported a reduction of melatonin (60 Hz, 20 μT) in persons with habitually low melatonin production but could not replicate that effect in a follow-up study. Wood *et al.*[4] observed reduced melatonin levels only when the magnetic field (50 Hz, 20 μT) was administered during the onset and rise of melatonin synthesis. This was not verified in the present study where exposure was accordingly set and where the field strength was 10-fold higher. But this does not exclude such an effect in case of an intermittent presentation.

In contrast to laboratory studies, field observations revealed almost consistently a reduction of urinary 6-OHMS excretion. Pfluger and Minder[26] observed this in railway workers who are usually exposed to 16.7-Hz magnetic fields (of an average of 20 μT) and Burch *et al.*[27] in electric utility workers. Similar results were obtained by Wilson *et al.*[11] whose subjects slept under electrically heated blankets and Karasek *et al.*[28] who treated 12 men with strong magnetic fields (workdaily repeated 20-minutes exposures to a 40-Hz field of 2.9 mT over 3 weeks).

Thus, it can be assumed that magnetic fields affect melatonin synthesis only after repeated exposures. This then explains the absence of any effect in the present as well as in other laboratory studies. The only study which took that in account was performed by Graham *et al.*[29] who exposed 30 healthy young men during 4 consecutive nights to intermittent magnetic fields of 28.3 μT. They determined a

slight instability of excreted 6-OHMS over time which might indicate a possible cumulative effect.

Infrared radiation: Infrared radiation did not affect melatonin synthesis but did influence rectal temperature and heart rate. The maxima and the amplitudes of both these variables increased considerably as compared to the control situation (Figs. 2, 3, Table 3, p=0.026, p=0.016).

The effect of melatonin on rectal temperature and the link of the latter with heart rate was repeatedly studied and verified[20]. The rise and decay of melatonin production is followed by an inversely shaped course of rectal temperature[14]. Suppression of melatonin results in an elevation and oral administration of melatonin in a reduction of rectal temperatures[15, 30]. A counter regulation seemed therefore possible, i.e. that melatonin production increases in response to the elevated temperature. This is, however, not supported by the present data.

Infrared radiation did not affect the temporal parameters of melatonin synthesis, whereas the nadirs of rectal temperature and of heart rate were significantly delayed. The increased distance between the nadirs and the acrophase of melatonin (temperature p=0.016, heart rate p=0.078) indicate an internal dissociation rather than a shift of the circadian system and thereby a pure thermophysiological response.

Conclusion

If applied during the time period where melatonin levels usually rise, even moderate bright light caused a delay of melatonin synthesis. The shorter delays of the nadirs of rectal temperature and heart rate indicate an internal dissociation, which might enhance the dissociation that anyway occur during shiftwork and accelerate the adverse effects on health. Apart from bright light, the other stressors are unlikely to affect the circadian rhythm. Infrared radiation caused pure thermophysiological effects (elevated rectal temperature and heart rate). Concerning extremely low-frequency magnetic fields there are no acute effects but it is assumed that the organism is prone to cumulative effects that become evident only after repeated exposures. To answer this question requires the execution of accordingly designed studies.

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Industrial Health 2002, **40**, 328–334 **Original Article**

Correlations between Workplace Protection Factors and Fit Factors for Filtering Facepieces in the Welding Workplace

Don-Hee HAN

Department of Industrial Health and Safety, Inje University, Gimhae, Gyeongnam, 621-749, South Korea

Received October 29, 2001 and accepted July 1, 2002

Abstract: Workplace protection factor (WPF) means a measure of the actual protection of respirator provided in the workplace when correctly worn. While fit factor (FF) represents a quantitative measure of the fit of a particular respirator to an individual and it is determined in the laboratory. To evaluate the relationship between WPF and FF is very important since FF may or may not be taken advantage of estimating WPF. Outside and inside Fe concentrations for three brand N95 filtering facepieces were collected on 14 workers/three respirator combinations in the welding workplace. The WPF measurements on the samples of the three respirator brands worn by 14 workers were observed to range from 2.2 to 132.9 with a geometric mean of 15.9 and a geometric standard deviation of 2.63. Respirator performances as measured by the WPF differed significantly among different respirator brands ($p<0.05$). In this study, correlations were found between the WPF measurements and the FF data for all samples of the three respirators ($R^2=0.38$). The percentage of Fe particles having a smaller fraction than 1.1 μm diameter was observed as 71.6% of the total.

Key words: Workplace protection factor, Fit factor, Welding workplace, N95 respirator

Introduction

Respirator fit testing is desirable before entering hazardous working environments to ensure that the respirator worn satisfies a minimum fit, and that the user knows when a respirator fits properly[1], although many Asian countries, including Korea, do not have fit testing regulations. The fit of a respirator can be determined by qualitative or quantitative methods. Quantitative fit test (QNFT) methods provide an objective and numerical basis by measuring a fit factor (FF)[2]. A perfect fit, which eliminates leaks, gives a fit factor of infinity; the worst possible case gives a fit factor of unity. QNFT, using a pass/fail level of fit factors, can help select a respirator that provides an appropriate level of protection to a worker as long as he/she wears it. In the past fit testing was commonly applied to an elastomeric respirator and a disposable respirator with HEPA filter. Because of the problem of the fit testing method, or the characteristics of a non-HEPA filter, a disposable respirator with non-HEPA filter could not be generally fit tested[3]. The new negative pressure air purifying particulate filter test criteria changed with respirator regulations in 42 CFR Part 84 in the United States[4] and the most penetrating sized aerosols are used in testing. Also, new technology now makes it possible to fit test N95 disposable respirators certified by the new test criteria, that could not be fit tested before. The N95 Companion® (TSI, USA) works with the Portacount Plus® to fit test even Class-95 filters including Class-99 and Class-100 filters[5]. Since the N95 disposable respirators were introduced into the market place, many questions regarding whether they, especially the disposable filtering facepieces, need to be fit tested have been raised. Recent study demonstrated the need for fit testing to screen out those types of respirators that do not fit well and do not provide the necessary level of protection[6].

Workplace performance of respirators was assessed by

*To whom correspondence should be addressed.

determining workplace protection factors (WPF) that are defined as "a measure of the protection provided in the workplace by a properly functioning respirator when correctly worn and used"[7]. WPF is difficult to determine because of difficulties of sampling and analysis in the workplace. Compared with WPF, FF determined in the laboratory are easy to measure. Therefore questions are raised about that FF can be used as benchmarks to provide some indication of the respirator's workplace performance. In other words, are there any correlations between FF and WPF? Up to now unfortunately no studies have been reported or found in the literature that identified such correlations. Those that have attempted to identify such correlations have been unsuccessful[8–11].

Meanwhile although a large portion of disposable filtering facepieces used in Korea is domestic, not a small number of those type respirator filters is imported from foreign countries, especially, the United States. Most of the N95 filtering facepiece certified by the U.S. certification regulation (42 CFR 84) passes Korean certification regulations[12], since the particulate filter test criteria of the former is stricter than those of the latter. It is thus necessary for the N95 filtering facepiece to be selected as a subject in this study.

The main goal of this study was to provide data on the workplace performance for new type certified filters, the N95 filtering facepieces, in the welding workplace. The second goal was to evaluate the relationship between WPF and FF for the N95 filtering facepieces using Fe concentrations in the welding workplace.

Materials and Methods

Workplace setting and workers selection

The workplace setting for this study was the welding shop of Hyundai Mipo shipyard in Korea. Workers selected in the study were working as trainees looking to get their international welding license for six months after joining the company. They are trained in a homogenous group of various training courses with a scheduled term. During the sampling they were working with oxygen fuel gas flame welding with mild steel for eight hours a day no shift. 14 welders in this research participated as volunteers. A test subject participated once in a sampling for each respirator, a total of three times. Three samples for a worker were taken in a day during all normal activities (from 08:00 to 17:00).

Respirators

Three different manufactured brands of filtering facepieces imported from the United States were chosen for the test. They are all certified as N95 by the U.S. certification regulations (42 CFR 84) that the National Institute for Occupational Safety and Health (NIOSH) promulgated in July 1995. They were the 3M 8511® cup-shaped N95 particulate filter with one valve (3M Co., USA), called after this mask "A"; the MSA affinity foldable FR 200® without valve (MSA, USA), called after this mask "B"; and the Willson N95 10FL foldable particulates respirator without valve (dalloz safety™, USA), called after this mask "C".

Fit testing

Respirators were randomly assigned to the workers, and their fit performance when worn by a worker was assessed by quantitative fit testing (QNFT). The TSI Portacount Plus® (Model 8020; TSI Inc., USA) with the N95 Companion® was used to conduct the fit testing[5]. The N95 Companion® was recently developed to test N95 respirators. This instrument removes the aerosol particles of which size range is the most penetrating to the respirator filter. Only a very small aerosol particles that does not significantly penetrate the filter remains. Thus, when using the N95 Companion®, filter penetration is not a concern. The N95 Companion® is simply an accessory for the Portacount Plus®. While measuring fit testing this instrument is attached with the Portacount Plus® and the FF measurement is made by the Portacount Plus®. The test exercises used normal breathing, deep breathing, head movement (head up and down, side by side), talking (reading) and jogging in place.

Sampling and analysis description

The research protocol used for this study was patterned after other published respirator field study protocols[13–16]. A total number of 42 WPF samples were sampled from 14 subject workers for the three brand respirators respectively, that is, each worker/respirator combination. Tradition lapel sampling systems for WPFs were set up to provide simultaneous samples from the breathing zone both outside and inside the respirator. Outside respirator sampling (C_o) was carried out as total suspended particulates. Inside respirator sample (C_i) was collected by a probe inserted through the respirator at a location situated between the nose and the mouth. Each sampling train consisted of 37 mm 0.8 μm mixed cellulose ester filter (SKC Inc., USA) mounted in three-piece plastic cassettes and a polypropylene back up pad connected to a personal sampling pump (AIRCHEK®50; SKC Inc., USA) by 1/4 inch tygon tubing. A flow rate of pumps was approximately 2 L/min for all

outside and inside sampling. Sampling pumps were calibrated to this flow with a bubble flow meter (UltraFlo®, Buck Inc., USA) and were checked before and after each WPF sampling period. Sampling pumps in both sampling trains were always simultaneously started or stopped.

Field blanks (FB) were taken to measure potential contamination due to handling of the filters and cassette, field storage and analysis (i.e., to account for background contamination). The blanks were treated in the same manner as the C_o and C_i sampling except no air was drawn through the tubes (i.e., not attached to any pump). The sealed cassette was clipped to the worker in an area close to the breathing zone. At the end of the sample period, the FB cassette was reopened and closed, stored with the other sample, and sent in for analysis. Manufacturer blanks (MB) were unused filters loaded into an unused cassette. One set of unused cassettes and filters was randomly collected, and stored with other samples and sent to be analyzed without ever being opened. FB and MB were analyzed the same way as were the filters sampled during the tests.

After approximately 2 hours, the pump was stopped and the cassette was plugged. At the end of each working day, the cassettes containing samples collected were forwarded to the laboratory for analysis. Samples were extracted for Fe in accordance with NIOSH Method 7300[17] and outside facepiece Fe concentrations were analyzed by flame method of atomic absorption spectrophotometer (Model AA-680, Shimadzu Inc., Japan) while inside facepiece Fe concentrations, FB and one set of MB were graphite furnace method of the same instrument (Model GFA-4B, Shimadzu Inc., Japan). The limit of quantification of Fe for flame method of this analysis was 0.8 μg for sample and graphite furnace is 0.08 μg for sample.

To account for loss to the filter and cassette wall, the interior surface of each sample cassette was acid washed using by modified the other published research protocol[15]. The washed solute and an acid digest of the cassette filters were combined and analyzed.

WPFs and FFs measurement program at the welding workplace

FFs should be measured in the laboratory. Therefore before entering the workplace each subject was first fit tested in the nearest office in which there was no air exchange with the workplace. After fit testing was finished, outside and inside sampling tubes of the respirator were plugged not to introduce outside aerosol into the tubes. The subject moved immediately to the workplace without removing the respirators accomplished fit testing and sampling for WPFs continued during work time. Three different respirator samplings for a subject were conducted in one day.

Ambient particle size measurement

Four cascade impactor samples were collected to determine the size distribution of the ambient particles at various locations at the study site. Sampling locations were selected as close as possible to those positions where study participants were most frequently situated. The samples were collected by 8-stage Anderson Non-Viable Sampler® (Anderson Inc., USA). Sampling was conducted for approximately 5 hours at a flow rate of 28.3 L/min. This sampling gave impactor stage effective cut-off diameter of 9.0, 5.8, 4.7, 3.3, 2.1, 1.1, 0.65, 0.43 μm with a backup filter to collect particle $\leq$ 0.4 μm.

Statistical analysis

The data was analyzed with student t-test, analysis of variance (ANOVA) after data log-transformed and correlation analysis. These statistical analyses require that the data being analyzed be normally distributed. Outside Fe concentration (C_o), inside Fe concentration (C_i), WPF data, FF data and the log-transformed data of them were checked for normality by using the Shapiro-Wilk test in LogNorm® software[18]. Other statistical analysis was performed by using SAS® software[19].

Results and Discussion

Outside and inside Fe concentrations, WPFs and FFs

Outside- and inside facepiece concentrations were expressed only in term of elemental Fe. Analysis of FB and MB showed no level of quantification (<0.08 μg), so that correction for background contamination of the filters and handling was not necessary. Table 1 summarizes outside-, inside facepiece Fe concentrations, WPFs and FFs. Based on the normality test, all of the data was found to be lognormal distributed except for inside Fe concentrations for mask 'A'. Subsequently geometric means (GM) and geometric standard deviations (GSD) were calculated for all data. The outside Fe concentration ranged from 180 to 1,831 $\mu g/m^3$ with GM of 675 $\mu g/m^3$ for all 42 samples and outside Fe concentrations in different brands respirators were not significantly different. The inside Fe concentration ranged from 7 to 311 $\mu g/m^3$ with GM of 43 $\mu g/m^3$ for all samples. The GM of inside Fe concentrations for mask 'A' were found to have the lowest value of 26 $\mu g/m^3$ and that of mask 'C', the moderate value of 42 $\mu g/m^3$, that of mask 'B' being the highest value of 70 $\mu g/m^3$, but there was no significant difference statistically

Table 1. Fe concentrations, workplace protection factors (WPF) and fit factors (FF)

Mask	Worker No.	Concentration, $\mu g/m^3$		WPF	FF
		Outside	Inside		
A	1	455	9	50.6	200
	2	754	84	9.0	33
	3	1,343	102	13.2	21
	4	1,103	80	13.8	191
	5	232	12	19.3	70
	6	1,789	188	9.5	137
	7	1,310	147	8.9	42
	8	194	8	24.3	189
	9	930	7	132.9	190
	10	323	15	21.5	89
	11	626	8	78.3	102
	12	354	11	32.2	55
	13	593	15	39.5	49
	14	787	28	28.1	20
	GM	633	26	24.4	75
	GSD	1.97	3.35	2.27	2.27
	Median	690	15	22.9	80
B	1	385	37	10.4	20
	2	970	137	7.1	12
	3	1,120	280	4.0	7
	4	915	311	2.9	5
	5	370	11	33.6	8
	6	1,831	228	8.3	9
	7	1,581	110	14.4	168
	8	256	119	2.2	5
	9	966	204	4.7	47
	10	574	102	5.6	15
	11	412	7	58.9	87
	12	367	24	15.3	20
	13	675	36	18.8	103
	14	1,035	41	25.2	16
	GM	695	70	9.9	19
	GSD	1.83	3.33	2.6	3.13
	Median	795	106	9.4	16
C	1	584	76	7.7	18
	2	980	208	4.7	10
	3	1,260	20	63.0	76
	4	759	36	21.1	61
	5	277	13	21.3	26
	6	1,567	26	60.3	137
	7	1,685	234	7.2	50
	8	180	45	4.0	45
	9	917	14	65.5	130
	10	584	34	17.2	10
	11	480	31	15.5	38
	12	580	14	41.4	58
	13	702	81	8.7	40
	14	873	84	10.4	21
	GM	699	42	16.6	39
	GSD	1.85	2.56	2.60	2.27
	Median	731	35	16.4	43
All Samples	GM	675	43	15.9	38
	GSD	1.86	3.22	2.63	2.93
	Median	728	36	15.4	44

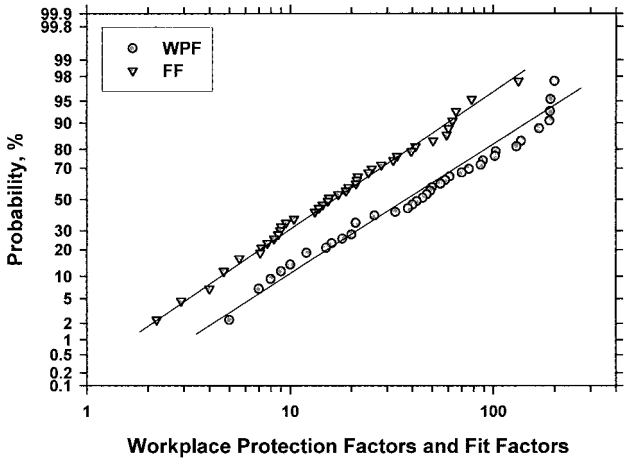

Fig. 1. Lognormal probability plots of WPF and FF for all samples of three respirators brands.

*Some symbols overlap one other to indicate fewer than 42 different measurements.

(P-value = 0.076). The reason that although there was a quantitative difference of inside Fe concentrations among brands, no significant difference appeared was thought to be the small sample size. None of the outside Fe concentrations as well as the inside Fe concentrations exceeded TLV of 5 mg/m^3 [20].

Lognormal probability plots of WPFs and FFs for three brands are presented in Fig. 1. The data of WPF and FF showed an approximate straight line indicating that lognormal statistics will give good estimates of mean and variability[21, 22]. The WPFs measured on all the samples of the three respirator brands were found to range 2.2 to 132.9 with GM of 15.9 and GSD of 2.63. The WPF of different brands was found to be significantly different ($P<0.05$). The FF for all samples ranged from 5 to 200 with GM of 38 and GSD of 2.93, and there was also significant difference in different brands ($P<0.05$). As shown in the graph, the FF was found higher than the WPFs at the same probability. Fig. 2 shows comparison of GM of the WPF with GM of the FF of each respirator brand. Student t-test for log transformed data indicated that the WPFs were significantly smaller than the FF in all respirators ($P<0.05$). This is naturally expected result that WPF was considered the penetration of particulates through the filter media, while FF is determined by only faceseal leakage but no penetration through the filter media is considered. In addition to penetration differences, fit testing is performed under somewhat ideal conditions for wearing the respirator: limited wearing duration, special care for fitting, aerosol sizes different from the workplaces, different breathing rate etc.

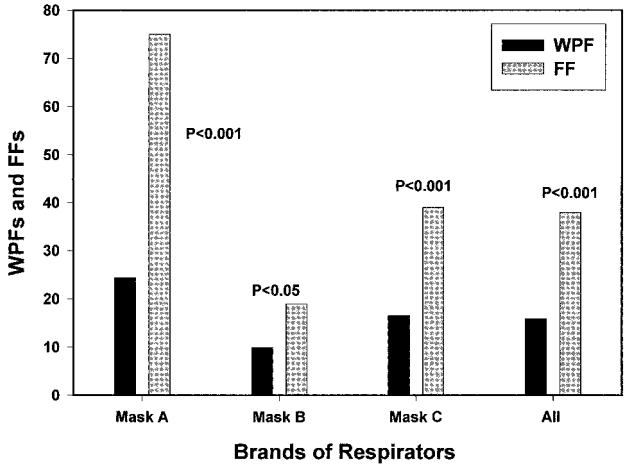

Fig. 2. Comparison of geometric means of WPFs and those of FFs with three respirator brands.

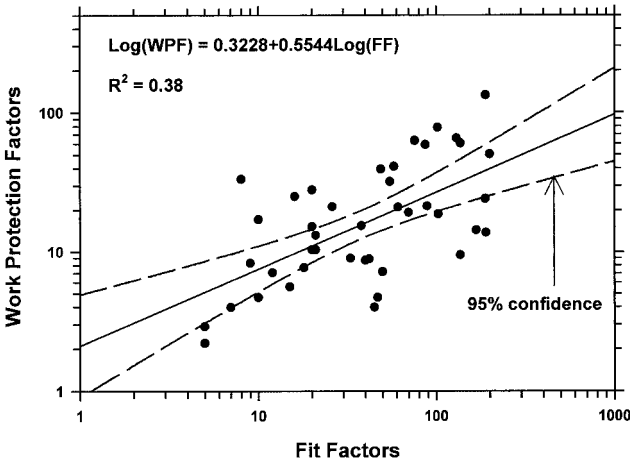

Fig. 3. Plots of workplace protection factors versus fit factors.

Table 2. Fe concentrations and size distributions for the impactor samples

Cut off diameter (μm)	Particle size range (μm)	GM concentration* (μg/m^3)	% of total	Cumulative % of total
>9.0	>9.0	41	9.7	100.0
5.8	5.8–9.0	17	4.0	90.3
4.7	4.7–5.8	12	2.8	86.3
3.3	3.3–4.7	15	3.6	83.5
2.1	2.1–3.3	16	3.8	79.9
1.1	1.1–2.1	19	4.5	76.1
0.65	0.65–1.1	48	11.4	71.6
0.43	0.43–0.65	81	19.1	60.2
<0.4	<0.4	174	41.1	41.1
Total		423	100.0	100.0

*Geometric means were calculated for 4 samples.

Correlation between WPF and FF

It can be thought that, the better the FF yields on workers/respirator combination, the better the WPF would be. Many researches were, therefore, conducted to identify correlation between WPF and FF, but most of all research showed no correlations between them[8–11] or weak correlations[23]. Correlation analysis was performed to evaluate the relationship between the WPF measured and the FF data for each respirator and for all respirators, using the WPF for the dependant variables and the FF for independents variables. As shown in Fig. 3, correlation between the WPF measurements and the FF results for all the samples of three brands respirators was found (R^2=0.38) and the linear regression model is significant at the 5% significance level. In the analysis for each respirator, mask 'A' was not strongly correlated (R^2=0.35), but mask 'B' (R^2=0.73) and mask 'C' (R^2=0.53) were both correlated between the WPF and the FF. The regression equation based on all samples of the three respirator brands was

$$\text{Log (WPF)} = 0.3228 + 0.5544 \text{ Log (FF)}$$

This result is not in agreement with the other research mentioned above. Correspondingly, although the data was very limited, this result suggests that, compared to the existing research, further more detailed researches should be necessary to identify whether or not WPF associates with FF for N95 filtering facepieces.

Particle size analysis results

Table 2 summarizes GM (N=4) of Fe concentrations and particle size analyzed by the impactor sampler. In this study, the Fe concentration by area sampling of 423 μg/m^3 was

lower than that by personal sampling of 675 $\mu g/m^3$ (See Table 1). Measurement of the particle size showed that approximately 90% of Fe particulate in the study is less than 9.0 μm. The percentage of Fe particulates mass having a smaller fraction than 1.1 μm diameter was found 71.6% of the total Fe mass. This result is not surprising since the fume size produced by the welding operations is, in general, less than 1.0 μm diamenter[24]. Rather, note that not a little fraction of particulate size of larger than 1.1 μm diameter was observed. This result indicates that fume particle consists of thousands of smaller, nearly spherical particles, or spherules, arranged in a complex branched-chain configuration after emitted and then become larger[25]. Or it is possible to explain that fume coagulates with existing dust after being emitted and then becomes larger.

On the other hand, according to Hinds and Kraske[26], aerosol penetration through face seal leaks was found to depend strongly on particle size, indicating that aerosol penetration through leaking increases dramatically with decreased particle size. The sizes of welding fume in this workplace were much smaller than the particle sizes produced by foundry operation[27] and steel mill operation[28]. For this reason, in this study face seal leaks, that is, a fraction of the total inside concentration may be a greater amount than the findings of research mentioned above. This result therefore suggests that more detailed research needs to identify the correlations between WPF and FF in small size aerosols produced by workplaces.

Conclusions

None of the outside facepiece Fe concentrations exceeded the TLVs of 5 mg/m^3 and no significant differences among the outside Fe concentrations of all samples of three brands respirators were found. The WPF measured on all the samples of three respirator brands worn by 14 workers ranged from 2.2 to 132.9 with GM of 15.9 and GSD of 2.63. Significant differences in respirator performance, as measured by the WPF were observed among different respirator brands ($p<0.05$).

In this study correlations were found between the WPF measurements and the FF data for all samples of three respirator brands ($R^2=0.38$). The percentage of Fe concentrations in particles having a smaller fraction than 1.1 μm diameter was found to be 71.6% of the total Fe concentration. The size of most particles produced by the welding fumes in this study were smaller than those in the foundry and steel mill operations in which WPF were not correlated with FF in the previous other studies. These results imply that more detailed study is necessary to evaluate the relationship between WPF and FF in small size aerosols such as welding fume.

Acknowledgements

The author is grateful to Yoon-Sok Jeong, a graduate student, Dept. of Industrial Health and Safety, Inje University, for participating in experiment. This research was supported by a grant from the Inje Research Foundation 2000.

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Industrial Health 2002, **40**, 335–344 **Original Article**

Increased Medication Use in a Community Environmentally Exposed to Chemicals

Rosemarie M. BOWLER[1]*, Sabine GYSENS[1], Christopher HARTNEY[1], Long NGO[2], Stephen S. RAUCH[1] and John MIDTLING[3]

[1] San Francisco State University, Department of Psychology, San Francisco, California 94132, USA
[2] Harvard University, School of Public Health, Department of Biostatistics SPH2, Boston, MA 02115
[3] University of Illinois at Rockford, Family & Community Medicine, Rockford, Illinois 61107, USA

Received March 5, 2001 and accepted July 29, 2002

Abstract: An epidemiological health study compared the health status of residents of a town exposed to an accidental Catacarb chemical release from an adjacent oil refinery, with the health status of demographically similar residents of an unexposed town in the region. Few studies of Catacarb's effects on humans exist; however, animal studies have shown it to be a respiratory, gastro-intestinal, dermatological and visual irritant. As part of the study, health questionnaires assessing pre- and post exposure symptoms, illnesses and medication use were mailed to residents in both towns. Medication use is sometimes reported to be a more objective and reliable measure of health outcomes[1)]. The current paper compared medication use of exposed and unexposed residents. Significant increases after exposure were found in the use of the following medications: antacid, asthma medication, cough and cold medication, eye medication, headache medication and sleep medication. These increases were consistent with reported symptoms, albeit of greater magnitude; no increase in medication use for other illnesses was reported. Medication use in this sample was consistent with patients' report of symptoms and may be a better measure of outcome.

Key words: Catacarb, Diethanolamine, Potassium metavanadate, Potassium-carbonate, Medication use, Symptoms, Illnesses, Chemical exposure

Introduction

Chemical accidents have been increasing phenomena in the United States, concurrently with the rising gasoline production in the last decade[2)]. Oil refineries are frequently located adjacent to poor residential communities[1, 3)]. Residents studied in these communities have been shown to suffer adverse health effects following chemical accidents. One such chemical accident occurred in 1994 in the area of Crockett, California, situated adjacent to and downwind from a major oil refinery. A regeneration tower developed a hole, which resulted in a 16-day accidental release of 200 tons of Catacarb, a chemical solution used commonly by refineries to separate carbon monoxide and carbon dioxide from hydrogen.

Catacarb chemical composition

The major toxic chemicals in the 1994 Crockett Catacarb release were Diethanolamine (DEA), potassium metavanadate, potassium borate, and potassium carbonate.

Diethanolamine (DEA) is a highly toxic compound. No human studies are available on this compound but animal literature[4, 5)] shows that it causes kidney, liver and heart problems, anemia, decreased sperm motility, necropathy of the brain and spinal cord, demyelination changes, skin alteration and death at very high doses.

The oil refinery's Material Safety Data Sheet (MSDS) for DEA indicates exposure limits of 0.46 to 3 ppm[6)]. The exposure of the Crockett community to DEA was at this limit and higher.

*Correspondence: 8371 Kent Drive, El Cerrito, CA 94530, USA
email rbowl@sfsu.edu

Vanadate is also a very toxic compound both when inhaled or when absorbed via other routes. The toxic effects and the toxicokinetics in humans and animals have been very well described and characterized in the literature[7–11]. Vanadate has been known to produce a wide range of effects on many systems in the body (respiratory, CNS, immune, cardiovascular, renal, and gastrointestinal systems) based on the dosage and the routes of exposure. In a study by governmental industrial hygienists, the exposure of five human volunteers to respirable vanadium pentoxide dust at concentrations of 0.2 mg/m^3 for 8 hrs caused severe upper respiratory tract irritation in the form of persistent productive cough[12]. Exposure to higher levels of vanadate (over 0.5 mg/m^3 for up to 2 weeks) has produced acute respiratory symptoms in humans which can persist for up to 2 weeks after removal of the exposure[13], and it has been reported that the toxic effects of vanadate in rats are cumulative[8].

Exposure to moderate levels of potassium carbonate by inhalation can cause irritation of the mucous membrane of the eyes and upper respiratory system. This chemical has irritant and caustic actions similar to that of potassium hydroxide, but less severe[14]. Damage to the mucous membrane of the eyes and the respiratory system is expected at the exposure levels incurred during the 1994 Catacarb release.

Exposure to potassium borate and diethanolamine at the projected levels are also expected to cause irritation to the mucous membrane of the eyes and the respiratory systems.

Description of the exposed community

The town of Crockett has 3300 predominantly working-class residents and is medically underserved. It neither has a medical clinic, nor a single physician practicing within the town limits. The exposure mobilized the town's community leaders who negotiated "Good Neighbor" agreements with the refinery, which resulted in the funding of an epidemiological health study of the community[3]. Following the accidental Catacarb release residents quickly reported such early acute symptoms as headache, diarrhea, fatigue, vomiting, disorientation, flu-like symptoms and general weakness. These acute adverse health symptoms enabled the town leaders to also successfully negotiate temporary refinery funding for a Good Neighbor Clinic (GNC) in their town for a period of approximately nine months following the release. Over 1500 people (including 1000 Crockett residents, representing almost one third of the town's population) were medically evaluated for health effects associated with the Catacarb release by the Good Neighbor Clinic. Consistent with the independent findings of the community epidemiological study[3], GNC physicians also reported new onset of upper respiratory, eye, gastrointestinal, and neurological conditions, as well as psychological problems, including anxiety, depression and sleep disturbance.

The existing literature on medication use and chemical exposure is relatively scarce. However, a few reports exist, linking increased medication use to chemical exposures. Dayal, *et al.*[1] found increased medication use for breathing-related symptom reports and hydrofluoric acid exposure from an oil refinery in a community in Texas. Doucet[15] postulated that the Desert Storm Syndrome is an example of multiple assaults upon the body's immune system with subsequent increases in symptoms and in medications. Pisati, *et al.*[16] report an association of exposure to toluene diisocyanate (TDI) with increased and worsening asthmatic symptoms and an increased need for medication. Two independent studies of toxic waste landfills both showed adverse health effects and increased medication use in nearby community residents[17, 18]. According to Dayal[1], medication use may be a more objective and reliable measure of health outcomes in exposed populations. In his 1994 study of exposure to hydrofluoric acid following a chemical spill in Texas, he found significant correlations between medication use and exposure, and between symptom reports and medication. For our study, analysis of the available medication data from the larger epidemiological study was undertaken. The current paper compares medication use in Catacarb exposed and unexposed residents.

Materials and Methods

A community health study was requested by the exposed town residents within one month of the Catacarb release and successfully negotiated with the refinery as one of their Good Samaritan agreements. The study took place six months after the release (Labor Day 1995). As noted above, the release was caused by a refinery regeneration tower, which developed a hole and, for 16 days, spewed toxic material into the atmosphere that drifted toward the nearby town of Crockett. As the release worsened and residents noticed sticky brown deposits on cars and houses in the town, they also developed health complaints forcing the refinery to temporarily shut down its operations.

In a previous paper[3], the authors compared prevalence rates of symptoms and illnesses in the exposed population to those of a carefully selected nearby non-exposed town, similar in size and demographic characteristics based on the US Census data for the region (Santa Venetia).

Table 1. Demographics and comparisons for exposed & non-exposed towns*

	Exposed		Non-exposed	
	Range (n)	Mean ± SD	Range (n)	Mean ± SD
Age	18–91 (509)	51.5 ± 15.4	19–90 (655)	53.0 ± 15.1
Number in household	0–7 (496)	2.2 ± 1.1	0–7 (640)	2.3 ± 1.1
Number of children	0–6 (495)	1.5 ± 1.3	0–9 (643)	1.7 ± 1.5

	Exposed	Control	
	% (n)	% (n)	p-value
Gender			0.099
Male	46.4 (236)	41.5 (272)	
Female	53.6 (273)	58.5 (383)	
Age categories			0.144
18–39	24.6 (125)	20.9 (137)	
40–49	25.5 (130)	24.9 (163)	
50–62	25.1 (128)	23.7 (155)	
63+	24.8 (126)	30.5 (200)	
Ethnicity			0.014
White	91.2 (464)	95.0 (621)	
Non-white	8.8 (45)	5.0 (34)	
Education			0.006
High school or less	28.3 (144)	21.1 (138)	
Some college	30.8 (157)	30.1 (197)	
Associate of arts or higher	40.9 (208)	48.9 (320)	
Salary			0.033
$0–19999	27.0 (127)	31.2 (199)	
$20000–29999	20.6 (97)	17.9 (114)	
$30000–49999	34.4 (162)	28.3 (180)	
$50000+	18.0 (85)	22.6 (144)	

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Demographic characteristics of exposed and non-exposed residents are shown in Table 1. The non-exposed town's residents were similar to those of the exposed town's residents in age, number in household, number of children, and gender. However, the non-exposed sample was slightly more educated, higher salaried and included slightly more whites than the exposed town. Baseline prevalence rates (prior to exposure) for illnesses and health symptoms were similar in both groups.

Procedure

Once the study was funded, informational meetings were held to educate the residents of both towns on the study's value and the importance of their participation. Confidential health questionnaires were mailed to each household and residents were asked to complete one set of questionnaires for each of the two primary adults. Customary survey methods, including follow-up letters and telephone calls, were employed to enhance response rate, and are described in more detail in Bowler *et al.*, 1997[3]. Identical questionnaires were used for both the exposed and non-exposed towns with the exception of questions pertaining to exposure variables. In all 3333 questionnaires were mailed, 1523 questionnaires to exposed households and 1810 to non-exposed households. The overall response rate was 43.1%: 38.9% for the exposed group and 46.6% for the non-exposed group.

One month after the completed questionnaires were received, a randomly selected sample of 200 respondents from both towns received a brief second questionnaire eliciting a further checklist identification of representative symptoms after the release in order to assess the questionnaires' reproducibility. Symptom reporting was found to be reliable in terms of both the type and the number of symptoms. Moreover, medical records of a randomly selected group of 35 respondents available from the GNC established community clinic were reviewed to assess

Table 2. Individual items of symptom checklist

Respiratory problems (N=3)	Depression (N=2)
Shortness of breath on exertion	Feeling depressed
Wheezing or whistling in your chest	Noticeable change in your personality
Cough	
	Anxiety (N=3)
Dermatological problems (N=1)	Feeling irritable
Skin Rashes	Feeling anxious
	Noticeable change in your personality
Visual problems (N=3)	
Blurred vision	Sleep disorder/fatigue (N=3)
Dark vision	Feeling tired more easily
Dim vision	Sleeping more than usual
	Difficulty falling asleep
Headaches/chemical sensitivity (N=3)	
Headaches at least twice a week	
A lower tolerance for alcohol	
Nausea not caused by something you ate	

concordance of patients' symptom reports and report of medication use with physician's medical records notations.

Health questionnaire

In addition to general demographic items, the questionnaire included a checklist of symptoms found in other studies to be sensitive to chemical exposure[19]. Table 2 shows individual items contained in the symptom categories of respiratory, dermatological, visual, headache/chemical sensitivity, depression, anxiety and sleep disorder. Participants were asked to indicate for each item whether or not they had experienced that particular symptom during the previous month (in this case five to six months after exposure). Responses were summed for each category. In addition, two subscales for depression and anxiety from the Brief Symptom Inventory (BSI) by Derogatis[20] were included. Scoring of the two BSI scales followed the guidelines in the manual.

The questionnaire also included 'doctor diagnosed' illnesses and inquired if any of these illnesses had worsened within the past six months. (The chemical release had occurred immediately prior to Labor Day 1994 and the questionnaires were collected 6 months after Labor Day, making the 6 month time frame a convenient unit of time for optimal recall.) New onset illnesses were indicated by the date of diagnosis and worsened illness (for those who had been diagnosed prior to the release) was indicated by the illness status after the release. Illnesses typically associated with chemical exposure (respiratory, dermatological, eye and immunologic)[21–28] were included, as well as other illnesses.

Medication use was investigated and medication categories were derived after consultation with three physicians knowledgeable in the areas of both chemical exposure and medications. All major body systems were considered and especially medications, both over the counter and by prescription, relevant to chemical exposure were included. The latter list specifically included medications for respiratory, dermatological, eye and immunologic conditions. Medication use for anxiety and depression was also assessed. Respondents were also asked to indicate if their medication use had changed (new and increased use) in the post-release period.

In terms of exposure characteristics, exposed participants were also asked if they had observed any brown sticky deposits (residuals of Catacarb) on either their car or house, and whether they gardened during the 1994 Labor Day weekend, the days of the highest levels of Catacarb release.

Estimates of exposure concentrations of the components of Catacarb were determined by a board-certified toxicologist, based on the estimates provided by a refinery company risk assessment[29]. For estimated human exposure to Catacarb components in the Crockett accident, please refer to Table 3.

Statistical analysis

Questionnaires that remained incomplete after repeated telephone contacts or were otherwise invalid were excluded in the final analysis.

Demographic variables for the two towns were analyzed with unpaired t tests and chi-squares. A linear generalized estimating equation (GEE) adjusting for gender, education and race was used to analyze the outcome variables. The

Table 3. Estimated human exposure to catacarb components during 16 day release*

Parameters	Components of catacarb			
	Diethanolamine (DEA)	Vanadate	Potassium borate	Potassium carbonate
Approx. component proportions of catacarb	2–15%	0.25–0.33%	5–7%	20–25%
Average air concentration (mg/m^3)	0.52	0.056	0.151	0.6
Amount released in 16 days (kg)**	15422	526	10886	40823
Estimated amount inhaled per day (mg)***	6.73	0.74	1.95	34
Estimated amount inhaled per 16 days (mg)***	108	11.9	31.3	538

*Estimated amount of catacarb released to the environment in 16 days=181436 kg. **Estimate based on average concentration of Catacarb of 10.4 mg/m^3. ***Estimated based on projections by the refinery risk assessment (Montgomery Watson, 1995, March) of the total amount of air breathed per day for 70 kg/adult=12.9 m^3/kg.

Table 4. Symptom categories and BSI binary scales analysis using generalized estimating equation adjusted for gender, education and race*

	Presence of symptoms				
	Exposed (N=509)	Non-exposed (N=655)	Estimated adjusted		
Domain	% (n)	% (n)	odds ratio	95% C.I.	p-value
Respiratory	61 (310)	35 (230)	2.9	(2.2–3.7)	0.0001
Dermatological	24 (121)	14 (89)	2	(1.4–2.7)	0.0001
Visual	31 (158)	12 (77)	3.2	(2.4–4.5)	0.0001
Headaches/chemical sensitivity	53 (270)	31 (200)	2.7	(2.1–3.5)	0.0001
Depression	45 (227)	31 (206)	1.9	(1.4–2.4)	0.0001
Anxiety	55 (282)	41 (266)	1.9	(1.5–2.5)	0.0001
Sleep disorder/fatigue	72 (365)	53 (349)	2.3	(1.8–3.1)	0.0001
BSI-Anxiety	50 (254)	38 (249)	1.7	(1.3–2.2)	0.0001
BSI-Depression	44 (222)	38 (252)	1.3	(1.0–1.6)	0.0668

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specification of the working correlation matrix is exchangeable to take into account the within household correlation factor. This factor is used in the estimation to obtain the robust point estimates and 95% confidence intervals of the variables of interest. The estimated adjusted odds ratios and risk difference ratios were computed and 95% confidence intervals were used for the presence of medication use before and after the release, and for medication use by exposure variables.

Results

The questionnaire mailing yielded 509 usable questionnaires from the exposed and 655 from the non-exposed towns. As can be seen in Table 1, the sample was primarily white, working middle class and slightly more educated than the general population. The observed increase in symptoms and illnesses in the exposed population are reported elsewhere[3)]. In summary, after controlling for the effects of gender, education, race and household cluster, post-exposure increases ($p<0.05$) in respiratory, visual, and dermatological symptoms were found (O.R. 1.3–3.0), as well as new onset of acute and chronic bronchitis (Risk difference 6.7%, 14.3%), asthma (O.R. 4.6), hay fever (O.R. 2.4), sinus trouble (O.R. 3.0), allergies (O.R. 4.7), skin rashes (O.R. 2.0), eye problems (O.R. 4.3), and bladder infection (O.R. 5.6). Table 4 shows the presence of a selection of reported symptom categories for both towns. Estimated adjusted odds ratios indicate that the exposed residents report experiencing significantly more symptoms than the non-exposed residents.

Table 5 shows the percent of doctor diagnosed illnesses for each town, both prior to and post-Labor Day. Residents who endorsed illnesses prior to the exposure were asked to report if their illnesses had worsened; residents who did not suffer from illness prior to the release were asked about new onset. Adjusted odds ratios and risk differences of residents'

Table 5. Percents of selected illnesses prior to exposure & percent worse (including new-onset) Using generalized estimating equation (GEE) adjusted for gender, education & race**

Questionnaire name	Exposed (N=509)		Non-exposed (N=655)		Measures of association for % worse			
	% Prior to Exposure (n)	% Worse (n)	% Prior to Exposure (n)	% Worse (n)	Type*	Measures of Association	95% C.I.	p-value
Allergies	6.1 (31)	24.8 (101)	8.1 (53)	6.8 (147)	Odds Ratio (GEE)	4.7	2.1–10.8	0.000
Acute bronchitis	4.3 (22)	6.7 (75)	5.6 (37)	0.0 (83)	Risk Difference	6.7	1.0–12.0	0.022
Chronic bronchitis	0.8 (4)	14.3 (28)	1.2 (8)	0.0 (33)	Risk Difference	14.3	1.0–27.0	0.039
Asthma	3.9 (20)	26.9 (67)	3.7 (24)	7.4 (54)	Odds Ratio	4.6	1.5–13.8	0.008
Skin rashes	4.7 (24)	16.3 (86)	6.0 (39)	8.7 (92)	Odds Ratio (GEE)	2.0	0.7–5.4	0.167
Eye problems	1.6 (8)	28.0 (75)	3.8 (25)	8.1 (74)	Odds Ratio (GEE)	4.3	1.6–11.6	0.004
Sinus trouble	1.6 (8)	28.9 (121)	1.1 (7)	14.1 (128)	Odds Ratio (GEE)	3.0	1.5–5.9	0.002
Hay Fever	6.3 (32)	17.1 (111)	7.3 (48)	8.0 (113)	Odds Ratio (GEE)	2.4	1.0–5.6	0.042
Sinus trouble	1.6 (8)	28.9 (121)	1.1 (7)	14.1 (128)	Odds Ratio (GEE)	3.0	1.5–5.9	0.002
Bladder infection	3.5 (18)	5.9 (68)	5.0 (33)	1.0 (96)	Odds Ratio	5.6	0.6–49.4	0.161
Psychiatric disorder	2.4 (12)	24.1 (29)	1.8 (12)	0.0 (18)	Risk Difference	24.0	9.0–40.0	0.034

* When the sample size is small, the GEE model fails to converge. The adjusted OR of Relative Risk is the computed. If one of the cells has 0 outcome then the risk difference (excess risk) & the 95% confidence interval is reported together with the p-value from the Fisher's Exact Test. **Partially reprinted with permission from the publisher (Bowler, Ngo, Hartney, *et al.*, 1997).

worsening or new onset illnesses in the exposed town were between 2.0 to 24.0.

The authors have also reported elsewhere[3] on the good to excellent reproducibility coefficients of the questionnaires that were evaluated for the first and second responding for each town. This finding indicates better than adequate reliability of residents' self report. In addition to these reproducibility results, we have reported elsewhere on the medical review of independent physician case histories and notes, which indicated a high concordance between symptoms reported in the questionnaire and those reported to physicians during medical visits. Again, this would indicate good reliability of the self-report, especially where the exposed residents are concerned.

As noted above, medication use report may be an even more reliable measure of health outcome than self-report of symptoms. Therefore, medication use of both towns was evaluated and is shown in Tables 6a and 6b. Table 6a shows a similar relative risk for medication use prior to the release in the two towns, with odds ratios ranging between 0.92 to 1.22. Prior to the release, the exposed group reported the use of slightly more medication for respiratory and immunologic health problems but not for skin, psychiatric or other problems prior to the release. After the release, a dramatic increase in medication use for the exposed town is noted (Table 6b) with odds ratios ranging from 1.7 to 19, whereas this is not the case for the unexposed town.

Table 7 shows odds ratios for medication use in the exposed town in relation to the above mentioned exposure characteristics (seeing sticky brown deposits on car or house and gardening during Labor Day weekend). Odds ratios above 1.5 were found for respondents seeing the brown sticky substance on their cars for asthma, cough, cold, eye medicine use and for antacids use. Similarly, odds ratios above 1.5 were found for residents who reported seeing the substance on their house, for the use of cough and cold medicine, anti-depressants, antacid and sleeping medication.

Discussion

A review of the literature on the effects of Catacarb exposure on animals or humans has shown that the symptoms and illnesses reported here are typically found as sequelae of such chemical exposures. While immediate symptom and illness reports are frequently taken after chemical accidents, few studies have looked at medication use. It has been proposed by Dayal *et al.*[1] that medication use may be a more reliable and objective measure of health outcomes than symptom reports alone.

As noted above, the main findings of this study indicate that the residents of the exposed community reported significantly increased symptoms and illnesses. Moreover, they also reported a significantly increased use of several types of medications following the Catacarb release. These medications were usually prescribed to alleviate symptoms of upper respiratory, eye, gastrointestinal and neurological conditions, and their use was consistent with increased symptom reports in these domains by the exposed residents.

Table 6a. Medications: scale percents and adjusted* odds ratios for taking medications prior to catacarb release

	Exposed (N=509)		Non–exposed (N=655)			
	% Prior to labor Day	n	% Prior to labor Day	n	Adj odds ratio	95% C.I.
Asthma medicine	7.5	38	5.3	35	1.22	0.96–1.55
Cough medicine	22.4	114	19.5	128	1.10	0.95–1.27
Cold medicine	31.2	159	27.5	180	1.13	0.99–1.28
Skin medicine	24.8	126	22.7	149	1.08	0.94–1.24
Eye medicine	14.5	74	11.8	77	1.11	0.93–1.33
Headache medicine	47.7	243	44.4	291	1.11	0.98–1.25
Pain medicine	31.6	161	26.1	171	1.15	1.01–1.32
Depression medicine	3.5	18	3.2	21	1.02	0.73–1.42
Anxiety medicine	2.9	15	3.1	20	0.92	0.65–1.32
Sleeping pills	6.3	32	5.6	37	1.02	0.79–1.31

Table 6b. Medications: scale percents and adjusted* odds ratios for increased medication use (including new-onset) after catacarb release

	Exposed (N=509)		Non–exposed (N=655)			
	% Increased	n	% Increased	n	Odds ratio	95% C.I.
Asthma medicine	51.1	45	16.7	36	5.2	1.8–14.7
Cough medicine	44.4	144	10.1	139	6.6	3.5–12.7
Cold medicine	34.8	187	8.5	199	5.9	3.3–10.5
Skin medicine	36.8	133	6.9	146	7.8	3.8–16.1
Eye medicine	58.7	92	15.3	85	10.2	4.8–21.9
Headache medicine	44.1	247	5.3	284	14.4	8.2–25.4
Pain medicine	34.9	172	8.1	173	6.7	3.4–12.9
Depression medicine	44.0	25	26.9	26	2.3	0.7–8.0
Anxiety medicine	56.5	23	9.1	22	19.0	3.3–110.7
Sleeping pills	65.0	40	10.3	39	1.7	0.3–9.1

*Adjusted covariates: gender, education, & race.

The exposed residents in this study reported both a greater prevalence of new onset use of medication and/or a significantly increased use of existing medication to alleviate their symptoms. As can be seen in Fig. 1, odds ratios for respiratory, dermatological and eye medication use were even higher than those for corresponding symptoms and illnesses. It is known from the medical records review conducted as part of this study that residents' report on medication use have good concordance with the sample of medical records reviewed by physicians. Medication use thus appears to be at least consistent with symptom report. If considered a more objective and reliable measure of outcome, it would again indicate significant long-term negative effects, possibly to a greater degree than assumed on the basis of symptom report alone. It is noteworthy that the exposed residents did not report increases in use of medication for illnesses unrelated to chemical toxic exposure - arthritis; antibiotics; elevated blood pressure and cholesterol; cardiac illnesses; diabetes. The exposed residents also did not report taking more herbal medicines than the non-exposed group. Of particular note is the doubled prevalence rate for respiratory and eye medication use in residents who noted Catacarb deposits on their car and their house. It can be hypothesized that individuals who had direct contact with Catacarb residue incurred greater levels of exposure and thus were more affected by the toxic chemicals, than individuals who did not. The specificity of exposed residents' medication use for associated illnesses and symptoms is suggestive of the reliability of the exposed residents' responses. This also lends further credence to the validity of the reported results and would argue against a generalized inflated self-report of health complaints by the exposed.

In conclusion, this study shows that the residents who were exposed to the Catacarb release not only developed

Table 7. Odds ratios for increased medication use (including new onset) by exposure variables (endorsed vs. not endorsed) for exposed town only

Medications	Saw sticky substance on car N=509 OR 95% CI	Saw sticky substance on house N=509 OR 95% CI	Gardened during or after release N=509 OR 95% CI
Respiratory			
Asthma medicine	2.29	1.47	1.41
	0.96–5.46	0.77–2.80	0.72–2.76
Cough medicine	1.55	1.97	1.55
	1.00–2.40	1.28–3.02	1.03–2.34
Cold medicine	1.70	1.67	1.65
	1.15–2.53	1.16–2.41	1.16–2.36
Dermatological			
Skin medicine	1.39	1.37	1.36
	0.87–2.21	0.90–2.08	0.89–2.09
Visual			
Eye medicine	2.23	1.02	1.30
	1.13–4.39	0.51–2.02	0.77–2.20
Sinus & Head			
Headache medicine	1.22	1.16	1.04
	0.86–1.71	0.86–1.57	0.79–1.38
Pain medicine	1.26	1.11	1.35
	0.85–1.88	0.76–1.60	0.95–1.91
Psychiatric			
Depression medicine	1.30	2.15	1.68
	0.36–4.72	0.58–8.02	0.44–6.45
Anxiety medicine	0.91	0.75	1.39
	0.27–3.00	0.17–3.32	0.46–4.22
Sleeping pills	1.21	2.08	0.78
	0.44–3.29	0.86–5.06	0.31–1.95
Antacids	1.65	1.55	1.35
	1.08–2.52	1.08–2.24	0.97–1.87

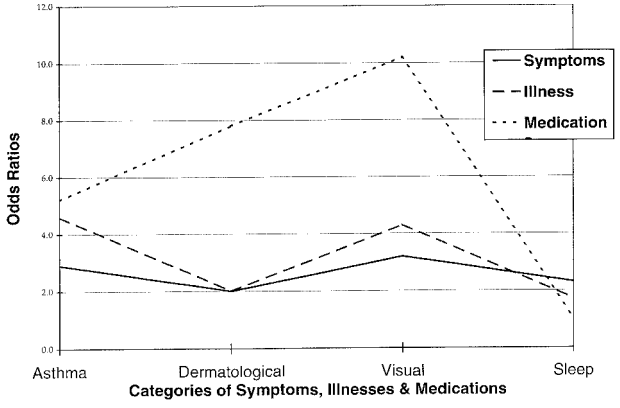

Fig. 1. Odds ratios by town for increased or worsening symptom, illness and medication use.

significantly more respiratory, dermatological, visual and psychiatric symptoms consistent with other laboratory reports of potential human health effects of the components of Catacarb, but also reported an increase in their medication use. This increase was only true for those medications used to alleviate the types of symptoms and illnesses reported to be associated with Catacarb exposure in human and animal studies. Future studies after chemical incidents might include pharmacy records with detailed information on dose and type of medication prescribed and purchased.

Acknowldegments

The participants from both towns in California, Crockett and Santa Venetia, are thanked for their efforts in making

this research possible. In particular the community leaders from the Shoreline Environmental Health Alliance, Ms. Virginia Bray and Ms. Patti Young, are thanked for trusting the senior investigator in conducting this health study. Also thanked are Dr. Stephen Borg, who assisted in constructing the medication categories, Dr. Ira Tager who helped in the design of the epidemiologic health study questionnaire, and Drs. James Cone and Ana Osorio, who reviewed the questionnaire. Dr. Jonathan Frisch and Dr. Mary McDaniel are thanked for assisting in obtaining funding for this study from Unocal Corporation. Dr. Margot Smith is appreciated for assisting with the choice of control town and reviews of the questionnaires. Kevin Lloyd is thanked for data entry and analyses as are many San Francisco State University students who assisted in mailing, data entry and data proofing. Michael Biel is thanked for the early management of this research and Ms. Hilary Foster is thanked for directing mailing and training staff in telephone contacts. Dr. Adam Duhan and Dr. David Greenly of the GNC are acknowledged for supporting this health study and assisting in obtaining medical records.

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Industrial Health 2002, **40**, 345–352 **Original Article**

Reproductive Toxicity of Carbofuran to the Female Mice: Effects on Estrous Cycle and Follicles

Prakash N. BALIGAR and Basappa B. KALIWAL*

Reproductive Toxicology Laboratory, Post-Graduate Department of Studies in Zoology, Karnatak University, Dharwad-580 003, INDIA

Received November 20, 2001 and accepted July 31, 2002

Abstract: Carbofuran, a systemic N-methyl carbamate pesticide was orally administered with the doses of 0.4, 0.7, 1 and 1.3 mg/kg body weight/day to normal virgin female Swiss albino mice for 30 days. The vaginal smear and body weight of mice were recorded daily and mice were sacrificed on the 31st day. Estrous cycle was effected by showing a significant decrease in the number of estrous cycle and the duration of each phases of estrous cycle with concomitant significant increase in the diestrus phase in 1 and 1.3 mg/kg/d carbofuran treatment when compared with that of control mice. There was a significant decrease in the number of healthy follicles and a significant increase in the number of atretic follicles in 1 and 1.3 mg/kg/d treated groups when compared with the control. The histologic observations of the ovary revealed the presence of less number of healthy follicles and more number of atretic follicles in high dose of carbofuran treated mice. There was a dose dependent decrease in the body weight. The ovary weight was also decreased significantly in 1.3 mg/kg/d carbofuran treatment. There were no significant change in the weight of the organs such as uterus, kidney, adrenal, liver, spleen, thymus and thyroid. These observed effects of carbofuran on the estrous cycle and follicles may be due to a direct effect on the ovary or the hypothalamo-hypophysial ovarian axis causing hormonal imbalance.

Key words: Carbofuran, Ovary, Follicles, Estrous cycle, Female mice, Toxicity

Introduction

Pesticides differ from any other chemical substances because they are deliberately spread into the environment. As a consequence, a great part of the human population may be exposed either in the general environment or in the working settings. The occupational exposure involving the manufacturing, and the use of pesticides, takes place mainly through dermal or respiratory route. While the environmental exposure, involving general population is mainly due to the ingestion of the contaminated foods and water. The environmental and occupational exposure determines the detrimental effect that this exposure could have on reproductive function. In women, if primordial follicles are destroyed extensively, they cannot be regenerated. This can cause premature ovarian failure, early menopause[1, 2]. The Carbofuran (2,3-dehydro-2,-2-dimethyl-7 benzofuranyl methylcarbamate), is a systemic N-methyl carbamate pesticide with predominantly contact and stomach action. It is mainly used as a soil applied chemical to control soil dwelling and foliar feeding insects and nematodes on a variety of agricultural crops, including maize, corn, rice, potatoes, alfalfa and grapes[3]. Carbofuran is a potent cholinesterase inhibitor and is highly toxic to humans and wildlife through the oral and inhalation routes of exposure[4]. It has been shown to affect the thyroid system in ewes, resulting in increased thyroxine concentrations[5]. Yousef *et al.*[6] have reported that the carbofuran decreased libido and sperm number in rabbits. The carbamate pesticides also have been recently reported in the induction of gonadal toxicity to female rats after chronic exposure to mancozeb and the administration of the sodium N-methyl dithiocarbamate

*To whom correspondence should be addressed.

inhibits the secretion of luteinizing hormone thus affecting the ovulation in rats[7, 8]. In view of the above findings, the present study has been undertaken to know the effect of a carbamate insecticide, carbofuran, on estrous cycle and follicular growth in virgin albino mice.

Materials and Methods

Technical grade carbofuran was kindly provided by Rallis India Ltd., Mumbai and dissolved in olive oil as a vehicle for oral administration. Laboratory bred virgin female Swiss albino mice aged 12–17 weeks, weighing between 24–28 g, showing regular 4–5 days estrous cycle were selected randomly from the breeding stock. The animals were housed in separate cages bedded with paddy husk and had free access to synthetic pellet diet "Gold Mohar" (Hindustan Lever Ltd., Mumbai) and water *ad libitum* throughout the study. The lighting schedule was 12:12 h light and dark cycle at a room temperature 26° ± 1°C. Doses were given below the acute LD_{50} level of intoxication[9]. Animals were divided into 5 groups having 10 animals in each. Carbofuran administered in doses of 0.4, 0.7, 1 and 1.3 mg/kg/d orally for 30 d to respective groups. The control group received an equal volume of olive oil. Daily vaginal smear and body weight were recorded throughout the experiment.

Estrous cycle

The phases of estrous cycle was determined by observing the vaginal smear in the morning (0800 h to 1000 h) as described by Cooper *et al.*[10]. Animals were sacrificed by cervical dislocation on the 31st day, 24 h after the final exposure and soon after the last vaginal smear.

Morphometric analysis of follicular growth

Ovaries of 5 animals in each group were taken for follicular growth studies. The estrous cycle stages of the five analysed ovaries displayed were in diestrus stage. The weight of ovaries of the animal nearest to the mean weight of the ovaries of respective group was selected. The ovaries were fixed in Bouin's fluid, embedded in paraffin, sectioned at 5 μm thickness and stained with hematoxylin and eosin. All serial sections of the ovary were counted for various stages of development of follicles as described by Moawad *et al.*[11], and Bolon *et al.*[12]. Follicles were classified according to Chen *et al.*[13] into small medium and large follicles. Healthy or atretic follicles were classified as described by Swartz and Mall[14].

In the present study, three classes of ovarian follicles were categorized using the relative cross sectional diameter of the follicle as measured from the outer margins of the granulosa cell layers. These quantitative criteria represent a substantial simplification of an elaborated grading system proposed by Pedersen and Peters[15], with eight stages and several sub stages to differentiate between primordial oocytes (Type 1) to antral follicle (Type 8).

1. Small follicles—(Pedersen and Peters Types 1-3b) consisted of an isolated oocyte or an oocyte surrounded by a partial or unbroken layer of granulosa cells.
2. Medium/growing follicles—(Pedersen and Peters Types 4-5b) has an oocyte surrounded by multilayered, solid mantle of granulosa cells.
3. Large/antral follicles—(Pedersen and Peters Types 6-8) were characterized by central oocyte and fluid filled space bordered by number of granulosa cells.

By using these criteria, mean diameters of follicles have been measured at approximately < 20 μm for small, 20–70 μm for medium and >70 μm for large follicles in mice. Follicles displaying the nucleus of the oocyte were measured by using a calibrated occular micrometer to avoid repeated counting. The maximum diameter and diameter at the right angle to it were used to obtain a mean diameter for each follicle. A follicle was considered to be undergoing atresia or to regressing whenever two or more pyknotic granulosa cells would be found in a single section or whether the oocyte showed signs of degeneration, such as fragmentation, loss of nuclear membrane, or thinning of cumulus oophorus as proposed by Osman[16].

Body and organs weight

The body weight gain was calculated on the basis of the weight taken on the 1st day soon after the oral administration considered as the initial body weight and the weight taken on the 31st d before cervical dislocation was considered as the final weight. Ovary, uterus, kidney, adrenal, liver, spleen, thymus and thyroid were dissected out, freed from adherent tissue and weighed to the nearest milligram. To ensure normalization of data for statistical analysis, organ weights were expressed per 100 g body weight.

Statistics

Statistical significance between the control and experimental data were subjected to analysis of variance (ANOVA) together with Dunnett's test. The Kruskal-Wallis test was used for the analysis of follicular counts. A probability of <0.05 was assumed to denote a significant difference.

Table 1. Effect of carbofuran on estrous cycle in albino mice

Groups	Treatment (mg/kg/d)	Number of mice	Number of cycles	Duration in days (M ± S.E.)				Diestrus index
				Proestrus	Estrus	Metestrus	Diestrus	
I	Control	10	5.9 ± 0.23	4.8 ± 0.29	7.6 ± 0.22	5.5 ± 0.22	11.9 ± 0.43	39.66
II	0.4	10	5.4 ± 0.16	4.4 ± 0.43	7.5 ± 0.27	5.3 ± 0.21	12.7 ± 0.33	42.33
III	0.7	10	5.2 ± 0.25	3.4 ± 0.51	7.1 ± 0.18	5.1 ± 0.18	13.9 ± 0.55	46.33
IV	1	10	4.3 ± 0.21*	2.9 ± 0.31*	6.2 ± 0.20*	4.6 ± 0.22	16.2 ± 0.54*	50.00
V	1.3	10	4.3 ± 0.3*	1.9 ± 0.32*	5.4 ± 0.34*	3.1 ± 0.23*	19.1 ± 0.53*	63.66

*=Significant $P<0.05$ compared to control.

$$\text{Diestrus index} = \frac{\text{Number of days with clear diestrus smear}}{\text{Total duration of treatment (Days)}} \times 100.$$

Results

Estrous cycle studies

The control mice exhibited regular estrous cycle and normal duration of each phases of estrous cycle. Treatment with 1.3 mg/kg/d carbofuran caused a significant decrease in the number of estrous cycle and duration of proestrus, estrus and metestrus with concomitant significant increase in the duration of diestrus phase. Treatment with 1 mg/kg/d carbofuran also caused a significant decrease in the number of estrous cycle and duration of proestrus and estrus with concomitant significant increase in the duration of diestrus phase. However, treatment with 0.4 and 0.7 mg/kg/d carbofuran showed no significant change in the number of estrous cycle and duration of each phases of estrous cycle. Diestrus index was also increased dose dependently following the administration of carbofuran (Table 1). However, the intoxicated mice were depressed and showed less running activity immediately after administration of carbofuran. This may be due to the inhibition of cholinesterase[17].

Morphometric analysis of follicular growth studies

The histologic observations of the control mice showed number of developing follicles, Graafian follicles, Corpora lutea and atretic follicles (Fig. 1). Treatment with 0.4 and 0.7 mg/kg/d carbofuran showed no significant change in the number of healthy and atretic follicles (Tables 2, 3). The histologic examination of the ovaries revealed developing follicles, corpora lutea and atretic follicles (Figs. 2, 3). Treatment with 1 and 1.3 mg/kg/d carbofuran caused a significant decrease in the number of healthy follicles with concomitant significant increase in the number of atretic follicles (Tables 2, 3). The histologic observations of the ovaries revealed fewer developing follicles, less number of corpora lutea and many atretic follicles (Figs. 4, 5) and the size of the ovaries was also reduced when compared with the control mice.

Body and organs weight studies

The mice treated with carbofuran for 30 d showed dose related toxicity in terms of gain in body weight. There is a significant decrease in the gain in body weight with 1.3 mg/kg/d carbofuran treatment. Treatment with different doses of carbofuran did not alter the weights of the uterus, kidney, adrenal, liver, spleen thymus and thyroid. However, there was a significant decrease in the weight of the ovary with 1.3 mg/kg/d carbofuran treatment (Table 4).

Discussion

Cyclic changes of the vaginal smear observed in the estrous cycle gives a reasonable index of the ovarian activity and its hormonal synthesis of estrogen and progesterone. The levels of these hormones are controlled by hypothalamus releasing gonadal hormones and pituitary gonadotropins[18]. The results obtained in the present study indicate that the control mice exhibited regular 4–5 d estrous cycle. Mice treated with 1 mg/kg/d carbofuran causes a significant decrease in the number of estrous cycle and the duration of proestrus and estrus with concomitant significant increase in the diestrus phase. However, treatment with 1.3 mg/kg/d carbofuran causes a significant decrease in the number of estrous cycle and the duration of proestrus, estrus, metestrus with concomitant significant increase in the diestrus phase. Diestrus index was also increased dose dependently in all the groups following the administration of carbofuran. Recently, similar results have been reported that the rats treated with a carbamate fungicide mancozeb causes a significant decrease in the number of estrous cycle and the duration of proestrus, estrus, and metestrus with a

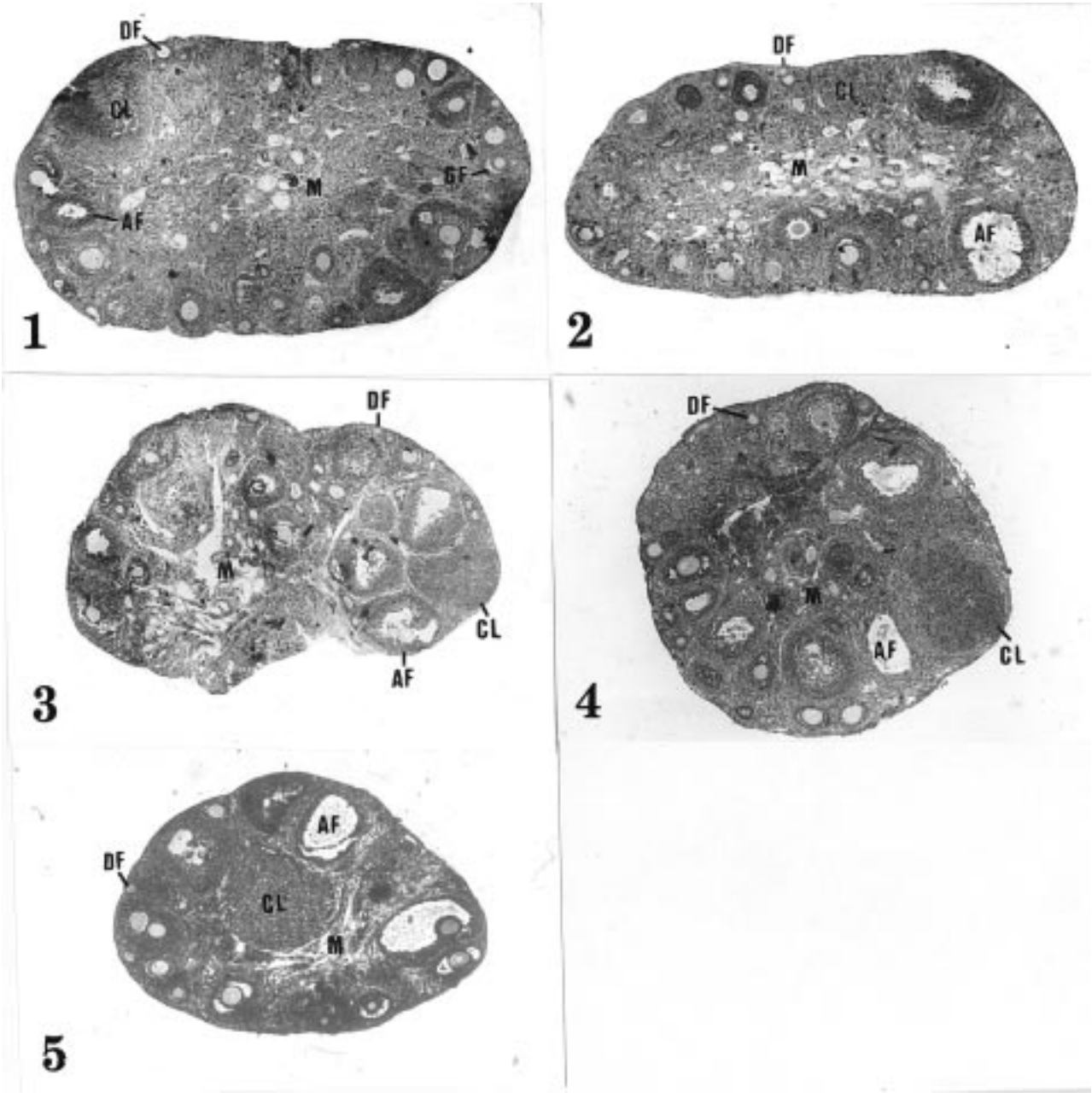

Fig. 1. Section of the ovary of control mice showing the presence of Graafian follicle (GF), plenty of developing follicles (DF), few atretic follicles (AF) and many corpora lutea (CL), M=Medulla; H & E × 30.

Fig. 2. Section of the ovary of mice treated with 0.4 mg/kg/d carbofuran for 30 days showing many developing follicles (DF), Corpora lutea (CL) and Atretic follicles (AF), M=Medulla; H & E × 30.

Fig. 3. Section of the ovary of mice treated with 0.7 mg/kg/d carbofuran for 30 days showing presence of developing follicles (DF), Corpora lutea (CL) and atretic follicles (AF), M=Medulla; H & E × 30.

Fig. 4. Section of the ovary of mice treated with 1 mg/kg/d carbofuran for 30 days showing presence of few developing follicles (DF), Corpora lutea (CL) and many atretic follicles (AF), M=Medulla; H & E × 30.

Fig. 5. Section of the ovary of mice treated with 1.3 mg/kg/d carbofuran for 30 days showing the presence of few developing follicles (DF), few small Corpora lutea (CL) and many atretic follicles (AF), M=Medulla; H & E × 30. The ovary is reduced in size.

Table 2. Effect of carbofuran on healthy follicles of the ovaries in albino mice

Groups	Treatment (mg/kg/d)	Number of mice	Number of healthy follicles by size classification (μm diameter); mean ± S.E.			Total no. of healthy follicles
			Small < 20 μm	Medium 20–70 μm	Large > 70 μm	
I	Control	5	206.6 ± 2.73	65.2 ± 2.73	7.2 ± 0.37	279.0 ± 2.97
II	0.4	5	207.6 ± 3.20	68.4 ± 2.73	7.2 ± 0.58	283.2 ± 3.44
III	0.7	5	204.6 ± 4.12	65.0 ± 1.64	6.6 ± 0.75	276.2 ± 4.44
IV	1	5	201.2 ± 3.02	56.0 ± 1.24	5.2 ± 0.58*	263.2 ± 2.96*
V	1.3	5	196.2 ± 1.96	52.2 ± 1.85*	4.2 ± 0.49*	252.6 ± 2.44*

*=Significant P<0.05 compared to Control.

Table 3. Effect of carbofuran on atretic follicles of the ovaries in albino mice

Groups	Treatment (mg/kg/d)	Number of mice	Number of atretic follicles by size classification (μm diameter); mean ± S.E.		Total no. of atretic follicles
			Medium 20–70 μm	Large > 70 μm	
I	Control	5	11.0 ± 0.89	2.2 ± 0.37	13.2 ± 1.07
II	0.4	5	10.8 ± 0.86	2.4 ± 0.24	13.2 ± 0.86
III	0.7	5	13.0 ± 1.00	2.4 ± 0.51	15.4 ± 1.21
IV	1	5	14.8 ± 1.02*	2.8 ± 0.37	17.6 ± 0.93*
V	1.3	5	21.4 ± 1.08*	3.8 ± 0.37	25.2 ± 0.80*

*=Significant P<0.05 compared to control.

Table 4. Effect of carbofuran on body and organs weight in albino mice

Groups	Treatment mg/kg/d	No. of mice	Body weight gain (g)	Relative weight/100 g body weight; mean ± S.E.							
				Ovary (mg)	Uterus (mg)	Kidney (g)	Adrenal (mg)	Liver (g)	Spleen (mg)	Thymus (mg)	Thyroid (mg)
I	Control	10	2.3 ± 0.21	28.81 ± 1.46	440.1 ± 7.87	1.16 ± 0.03	37.04 ± 3.04	5.26 ± 0.18	381.51 ± 5.66	173.14 ± 7.91	6.02 ± 0.64
II	0.4	10	1.9 ± 0.18	24.24 ± 1.30	443.68 ± 4.58	1.13 ± 0.04	34.01 ± 2.41	5.35 ± 0.21	396.99 ± 5.09	170.20 ± 10.59	6.77 ± 0.72
III	0.7	10	1.7 ± 0.33	25.10 ± 1.36	442.25 ± 3.71	1.12 ± 0.05	32.89 ± 2.39	5.57 ± 0.16	374.68 ± 8.13	162.52 ± 6.29	7.00 ± 0.51
IV	1	10	1.4 ± 0.31	23.48 ± 1.38	432.23 ± 3.16	1.04 ± 0.04	34.57 ± 1.99	5.22 ± 0.13	354.59 ± 9.83	156.30 ± 5.80	6.88 ± 0.49
V	1.3	10	1.0 ± 0.26*	18.84 ± 1.88*	435.62 ± 3.11	1.03 ± 0.04	31.84 ± 0.99	4.91 ± 0.43	362.50 ± 6.66	155.07 ± 7.78	8.06 ± 0.40

*=Significant P<0.05 compared to control.

concomitant significant increase in diestrus phase[7, 19]. Similar results have also been reported with other organophosphate pesticides treated animals[20–25]. In contrast, organochlorin pesticides like DDT, chlordecone, methoxychlore and dicofol showed a capacity to induce the persistent vaginal estrus, thereby affecting the number of estrous cycle resulting from the hormonal imbalance and prolonged estrus[26–30]. In the present study it has been shown that treatment with carbofuran showed prolonged diestrus and hence, carbofuran may not have estrogenic activity as it has been shown in the chlorinated pesticides treated animals. It has been reported that carbamate fungicide sodium N-methyl dithiocarbamate is shown to block the ovulation by inhibiting the secretion of LH in rat[8]. Since carbofuran is a carbamate pesticide, it may be possible that it would act on the level of hypothalamus to adversely affect the ovary, which in turn affects the estrous cycle and folliculogenesis due to the hormonal imbalance in estrogen progesterone ratio.

Plowchalk *et al.*[31] have reported that the quantitative assessment of follicle number is an indicator of the normal

function as well as toxic responses in the ovary. Follicles are the principle functional units of the mammalian ovary. The most important controllers of follicular development are follicle stimulating hormone (FSH) and luteinising hormone (LH) produced from the pituitary and the ovarian steroid estradiol produced by granulosa cells. Although all follicles are apparently exposed to the same fluctuations in these hormones, not all are equally responsive, some ovulate and others become atretic, indicating the presence of intragonadal regulatory factors which modulate the effect of these major hormones[32]. The present study revealed that the medium and total number of healthy follicles were significantly decreased with concomitant significant increase in medium and total number of atretic follicles in higher doses, but there was no effect on lower doses of carbofuran treated mice. Similar findings have been reported on the reduction of different types of healthy follicular stages with concomitant increase in the atresia in rats and mice treated with different pesticides. It has been reported that the chlorinated pesticides induces follicular toxicity in reducing the pool of healthy, large and medium sized follicles with increase in the atretic follicles[14, 29, 30]. It has also been reported that the administration of a number of organophosphate pesticides to adult rats increased the number of atretic follicles with concomitant decrease in the number of some of the follicular stages and total number of healthy follicles in dose and duration dependent manner[22–24]. Ataya *et al.*[33] have reported that the cyclophosphomide is found to inhibit the development of the antral follicles in rats, thereby increasing atretic follicles through interfering with hormonal ovarian follicular development and reduces estradiol. It has been shown that the ovarian andragen and inhibin secretion by follicles may be an important part in the regulation of FSH secretion and follicular growth[34]. McCann[35] has reported that a wide variety of pharmocologic agents that modify the neurotransmitter levels would act at the level of hypothalamus to adversely effect the reproductive function.

Treatment with carbamate fungicide sodium N-methyl dithiocarbamate is shown to block the ovulation by inhibiting the secretion of luteinizing hormone in rats[8]. Recently it has been reported that rats treated with carbamate fungicide mancozeb, showed a decrease in the number of healthy follicles with increased atretic follicles[7, 19]. In the present study, there is also a possibility that the decreased healthy follicles with concomitant increase in atretic follicles in mice may be due to affecting gonadotropin secretion via central nervous system mechanism, as it was observed in the rats with the following administration of dithiocarbamates[36]. In the present study there is also a possibility that the disruption in the estrous cycle, decrease in the healthy follicles with concomitant increase in the atretic follicles may result from the damage by toxicants at the level of hypothalamo-pituitary gonadal axis. It has been reported that the insecticides may destroy endocronologic Homeostasis, by suppressing GnRH release, may act directly on the gonadotropins to alter the gonadotropins synthesis and secretion or indirectly by altering the pituitary cell responsiveness to GnRH or gonadal steroids which result in the alterations in the levels of FSH and LH affecting the feed-back mechanisms[37–39]. Further evidence of hormonal imbalance is corroborated with as the mice shows continuous diestrus. Therefore, the reason may be due to the hormonal imbalance in any of the stages in hypothalamo-hyphophysial ovarian axis or by insensitising the follicular receptors to the available gonadotropins thereby led to the retardation of further development of surviving follicles into next successive follicular stages and also arrest of estrogen production which affects the estrous cycle or directly on the ovary by causing fibrosis[36, 40]. However, further investigation in this regard is essential to know the mechanism of action of carbofuran on follicular development and estrous cycle. Treatment with carbofuran showed dose related toxicity in terms of body weight. There is a significant decrease in the body weight gain in high dose of carbofuran treatment, as there may be suppression towards food and water intake. Although food and water intake has not been measured in this study, this may be one of the reasons for low weight gain and alteration in the estrous cycle. The ovary weight was decreased significantly with high doses of carbofuran treatment. Similar observations were made in rats treated with monocrotophos and have reported that decrease in weight and size of the ovaries is due to extensive fibrosis and atretic follicles[40, 41]. Treatment with carbofuran in different dose groups did not alter the weights of uterus kidney, adrenal, liver, spleen, thymus and thyroid. Similar results have been reported in other pesticide treated rats[7, 19, 30].

The alteration in the estrous cycle with prolonged diestrus and decrease in the healthy follicles with an increase in the atretic follicles in carbofuran treated mice may be due to the reduced synthesis of steroids in the ovary, causing imbalance in the estrogen: progesterone ratio. Whether the observed toxicity occurred as a result of direct effects upon the ovary or indirectly through the action on the hypothalamus and/or pituitary, or by desensitizing the ovary to gonadotropins cannot be ascertained from this study. Further investigation on the mechanism of carbofuran ovarian toxicity will be necessary.

Acknowledgement

The authors are grateful to the U.G.C. New Delhi, for financial support [Grant No. F.3-13/99 (SAP-II)]. Our thanks are due to Prof. S.A. Nevagi, Chairman, Post-Graduate Department of Studies in Zoology, Karnatak University, Dharwad for providing necessary facilities.

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Industrial Health 2002, **40**, 353–361 **Original Article**

Thermotactile Perception Thresholds Measurement Conditions

Setsuo MAEDA[1] and Hisataka SAKAKIBARA[2]

[1] Department of Human Engineering, National Institute of Industrial Health, 21-1, Nagao 6-chome, Tama-Ku, Kawasaki 214-8585, Japan

[2] Nagoya University School of Health Sciences, 1-1-20, Daiko-Minami, Higashi-Ku, Nagoya 461-8673, Japan

Received July 1, 2002 and accepted August 22, 2002

Abstract: The purpose of this paper is to investigate the effects of posture, push force and rate of temperature change on thermotactile thresholds and to clarify suitable measuring conditions for Japanese people. Thermotactile (warm and cold) thresholds on the right middle finger were measured with an *HVLab* thermal aesthesiometer. Subjects were eight healthy male Japanese students. The effects of posture in measurement were examined in the posture of a straight hand and forearm placed on a support, the same posture without a support, and the fingers and hand flexed at the wrist with the elbow placed on a desk. The finger push force applied to the applicator of the thermal aesthesiometer was controlled at a 0.5, 1.0, 2.0 and 3.0 N. The applicator temperature was changed to 0.5, 1.0, 1.5, 2.0 and 2.5°C/s. After each measurement, subjects were asked about comfort under the measuring conditions. Three series of experiments were conducted on different days to evaluate repeatability. Repeated measures ANOVA showed that warm thresholds were affected by the push force and the rate of temperature change and that cold thresholds were influenced by posture and push force. The comfort assessment indicated that the measurement posture of a straight hand and forearm laid on a support was the most comfortable for the subjects. Relatively high repeatability was obtained under measurement conditions of a 1°C/s temperature change rate and a 0.5 N push force. Measurement posture, push force and rate of temperature change can affect the thermal threshold. Judging from the repeatability, a push force of 0.5 N and a temperature change of 1.0°C/s in the posture with the straight hand and forearm laid on a support are recommended for warm and cold threshold measurements.

Key words: Thermotactile threshold, Vibration, Posture, Push force, Repeatability

Introduction

Neurological disturbances such as finger numbness or tingling, and impaired skin perceptions are often encountered in hand-arm vibration syndrome. In Japan, as a medical checkup of hand-arm vibration syndrome, the measurement of pain thresholds has been performed according to the Labour Standards Bureau Notification No.609, but measurement with a weighted syringe needle is a question under discussion due to the risk of infection. A new index for special medical checkup of vibration syndrome is required in place of the pain threshold measurement. Meanwhile, the usefulness of the thermotactile threshold has been reported as a diagnostic measure for evaluating the function of small sensory nerve fibers involving unmyelinated C fibers and myelinated A-σ fibers[1, 2, 4]. As pain threshold measurement is also expected to examine the dysfunction of small sensory nerve fibers, thermotactile threshold measurement can be a substitute for pain threshold measurement.

A deterioration in thermotactile thresholds has been demonstrated among vibration-exposed workers[2, 4] and thermotactile threshold testing is recommended to evaluate

*To whom correspondence should be addressed.

the function of small sensory nerve fibers[1]. It is, however, indicated that the thermotactile threshold is affected by various factors in the measuring conditions such as the reference temperature or skin temperature[3, 7, 11], the applicator size[3], the rate of temperature change[3, 6, 10], and the push force[6, 10]. With respect to the reference temperature or skin temperature, previous studies have shown that the repeatability of thermal testing is sufficiently accurate when the skin temperature of subjects is over 30°C[3, 11]. It is also reported that the thermal threshold is lower with a big probe than with a small one[3]. These two factors are physiologically fundamental measuring conditions.

Previous studies have also indicated that a 1°C temperature change[3, 6, 10] and a 2 N push force[6, 10] are recommendable. But the effects of the rate of temperature change and the push force were not investigated at once, nor was the effect of the push force itself examined fully. It is not clear whether these measurement conditions can apply to Japanese people, although many researches have been done to measure the thermotactile perception thresholds around the world.

In addition, Lindsell and Griffin[6] suggested that the measurement posture of flexion or rotation of the wrist during thermal measurements might result in compression of the ulnar or median nerve and affect the measured threshold. This means that nerves and blood vessels may be pressed in thermal measurement. Moreover, the posture of wrist flexion might possibly compress the vessels as well as the nerves in the wrist, which could reduce blood flow to the finger and deteriorate the nerve function.

Nevertheless, the effects of measurement posture on the thermal thresholds have not been investigated experimentally.

Therefore, the purpose of this study was to investigate the effect of posture, push force and rate of temperature change on the thermotactile threshold, considering these three conditions simultaneously, and to clarify suitable measurement conditions.

Subjects and Methods

Subjects

Eight male subjects participated in the present study. All subjects were healthy male students at the Human Factors Research Unit, Kinki University, who had no neuromuscular or vascular disorder and no previous serious injury to the upper extremities. Their mean age was 22.6 (SD: 0.7) years were, height 170.1 (6.0) cm and weight 62.2 (5.0) kg. The present series of experiments were conducted according to the safety recommendations and with the approval of the Human Experimentation Safety and Ethics Committee of the Department of Industrial Engineering, Kinki University.

Equipment specifications and procedure

Thermotactile (warm and cold) thresholds on the palmar surface of the distal phalanges of the right middle finger were measured with an *HVLab* thermal aesthesiometer[6]. The measurements were conducted in a soundproof room, where the room temperature ranged from 24 to 26°C, and the noise level was 35 dB(A).

Each subject was seated and relaxed in the room. Skin temperature of the right middle finger was measured with a temperature probe of the aesthesiometer. When the temperature was above 30°C, the temperature was set as the starting temperature of the applicator of the aesthesiometer. If not, the subject's finger was warmed beforehand to above 30°C with a heater, so that the starting temperature of all subjects was above 30°C (Mean; 34.4°C, SD; 1.2°C).

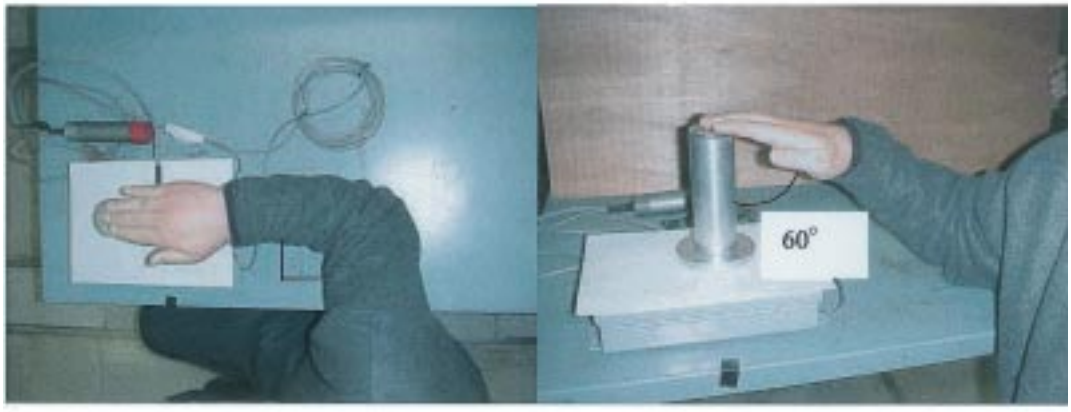
The right middle finger of the subject was put on the applicator, and then the temperature of the applicator was increased or decreased from the starting temperature at a rate of change which could be varied by mean of a software program. When the subject perceived warm or cold sensation and pressed the response button, the temperature was reversed and returned to the starting temperature. The applicator temperature was held at that temperature for 5 s and then began to increase or decrease at the same rate of change. An automatic test program repeated this process several times to establish the mean threshold levels of warm or cold sensation. In the present study, this process was repeated six times to obtain each warm and cold threshold respectively. The warm or cold threshold for each subject was then determined as the mean of the last four measurements, excluding the first two measurements that were regarded as trials. During the measurement, the push force on the applicator was controlled by the subject's visual feedback from electronic scales.

After each measurement under different measurement conditions, the subjects were asked about comfort during each measurement (+1 comfortable; 0 fair; and –1 uncomfortable).

Measurement parameters

In the present series of experiments, thermal thresholds were measured under different conditions of posture, push force applied to the applicator, and the rate of change of applicator temperature. The effect of posture was studied with three different postures: 1) straight hand and forearm laid on a support (posture 1), 2) the fingers and hand flexed

Posture 1 (Straight hand and forearm laid on a support)



Posture 2 (The fingers and hand flexed at the wrist with elbow placed on a desk)

Posture 3 (Straight hand and forearm without a support)

Fig. 1. Postures for thermotactile threshold measurement.

at the wrist with the elbow placed on a desk (posture 2), and 3) straight hand and forearm without support (posture 3) as shown in Fig. 1.

Push force was examined in four conditions, 0.5, 1.0, 2.0 and 3.0 N, because a 2 N push force has been recommended[6]. The applicator temperature was increased or decreased at a rate of 0.5, 1.0, 1.5, 2.0 and 2.5°C/sec, as some studies have suggested that a 1°C/s temperature change rate was recommended[3, 10].

The present study investigated these three measurement conditions, considering them simultaneously. As a result, the experiment conditions were 60 conditions (3 postures × 4 push forces × 5 temperature change rates). The subjects took part in 5 sets of 60 conditions with a rest for more than

Table 1. Mean threshold values and standard deviations in parenthesis for Posture 1

Rate of temperature change: °C/s	Push force: N	Day 1		Day 2		Day 3	
		Warm threshold (°C)	Cold threshold (°C)	Warm threshold (°C)	Cold threshold (°C)	Warm threshold (°C)	Cold threshold (°C)
0.5	0.5	37.51 (1.62)	31.86 (0.99)	37.56 (1.93)	31.14 (1.72)	38.48 (1.89)	31.36 (1.69)
	1	37.69 (1.70)	32.31 (1.34)	37.48 (0.94)	31.64 (2.03)	38.09 (1.77)	31.53 (1.57)
	2	37.28 (2.20)	31.63 (1.43)	37.48 (1.51)	31.79 (1.31)	37.98 (2.39)	31.55 (1.69)
	3	37.54 (1.79)	31.71 (1.78)	37.55 (1.22)	31.39 (2.17)	38.15 (1.69)	31.34 (1.62)
1	0.5	38.69 (1.95)	32.03 (1.28)	37.98 (1.87)	31.89 (1.66)	37.78 (1.60)	31.80 (1.35)
	1	37.85 (2.35)	32.40 (1.85)	38.33 (1.93)	32.36 (1.77)	38.39 (1.37)	32.40 (1.02)
	2	38.41 (1.93)	32.26 (1.01)	37.89 (1.90)	32.08 (1.32)	37.49 (2.24)	31.49 (1.79)
	3	37.88 (1.40)	32.60 (1.03)	37.90 (1.71)	32.25 (1.53)	37.89 (1.64)	32.26 (1.76)
1.5	0.5	38.29 (1.63)	31.25 (0.91)	39.31 (2.44)	30.91 (1.04)	38.99 (2.32)	30.80 (1.98)
	1	39.26 (1.34)	31.66 (1.46)	38.56 (1.79)	31.36 (1.15)	39.01 (1.94)	31.21 (1.50)
	2	38.16 (1.15)	31.55 (0.79)	38.48 (1.53)	31.36 (0.83)	38.74 (1.44)	30.89 (1.52)
	3	38.53 (0.96)	32.08 (1.09)	38.64 (1.46)	31.43 (0.96)	38.60 (1.20)	31.60 (0.93)
2	0.5	39.68 (2.41)	30.38 (1.34)	40.25 (1.87)	30.43 (1.74)	40.40 (1.98)	30.30 (1.70)
	1	39.11 (2.25)	30.61 (1.37)	39.84 (1.89)	30.81 (1.28)	39.68 (2.16)	30.29 (1.53)
	2	38.73 (1.83)	30.56 (0.78)	39.43 (1.90)	30.90 (1.70)	39.10 (1.75)	30.21 (1.57)
	3	38.88 (1.81)	30.34 (1.22)	39.61 (2.25)	30.48 (1.39)	38.79 (2.12)	30.25 (1.27)
2.5	0.5	40.44 (1.81)	31.43 (1.68)	39.68 (2.03)	31.06 (1.48)	40.44 (1.51)	31.49 (0.92)
	1	39.71 (1.48)	31.60 (1.70)	39.35 (0.59)	31.54 (1.70)	39.78 (1.11)	31.66 (1.47)
	2	39.30 (1.61)	31.30 (1.28)	39.09 (1.53)	31.34 (0.98)	39.65 (1.62)	31.29 (1.35)
	3	39.96 (1.37)	31.59 (0.83)	39.16 (1.29)	30.74 (1.16)	39.43 (0.61)	31.56 (0.99)

60 min between experiments in one day between 9:00 a.m. and 5:00 p.m. Accordingly it took 12 days to finish one series of experiments under the 60 conditions. To investigate the repeatability of each set of measurement conditions, all subjects took part in another series of experiments under 60 conditions on different 12 days, and repeated them again on another 12 days, so that all subjects underwent three series of experiments under 60 conditions on 36 days different.

Statistical analysis

Data analyses were conducted with SPSS for Windows, version 9.0. Repeated measures analysis of variance (ANOVA) was used to analyze factors of posture, push force, rate of temperature change and measurement day which may affect warm and cold thresholds. The repeatability of thermotactile thresholds on two different days under the same measurement conditions was evaluated with Pearson's correlation coefficients and r.m.s. errors[8]. The r.m.s. error was calculated from the following formula:

$$\text{r.m.s. error} = \sqrt{\sum_{i=1}^{n} (Xi - Yi)^2}$$

where Xi are the data for day X, Yi are the data for day Y, and *i* is the subject number. Pearson's correlation coefficient was greater and the r.m.s. error was smaller, and the measurement conditions were thought to have a good repeatability of thermal threshold measurements. Differences were considered statistically significant at $p<0.05$.

Results

Warm and cold thresholds

Tables 1, 2 and 3 show the results of mean and standard deviations of warm and cold thermotactile thresholds obtained under different measurement conditions.

Thresholds tended to be higher in the order posture 1, posture 2 and posture 3. The standard deviations of thermal thresholds also tended to increase in that order. With a warm threshold a small value means more sensitivity to warmth, whereas with a cold threshold a large value implies more sensitivity to cold. The means and the standard deviations of thermal thresholds were likely to be the smallest when the right straight hand and forearm was laid on a support (posture 1). As the rate of change of the applicator temperature was increased, the warm threshold tended to increase a little. With an increase in the push force, the warm or cold threshold also tended to decrease or increase

Table 2. Mean threshold values and standard deviations in parenthesis for Posture 2

Rate of temperature change: °C/s	Push force: N	Day 1		Day 2		Day 3	
		Warm threshold (°C)	Cold threshold (°C)	Warm threshold (°C)	Cold threshold (°C)	Warm threshold (°C)	Cold threshold (°C)
0.5	0.5	37.65 (2.68)	30.48 (2.30)	38.55 (1.96)	31.23 (1.96)	38.76 (2.94)	30.28 (2.45)
	1	38.14 (1.59)	31.30 (2.37)	38.03 (1.95)	30.81 (1.83)	38.03 (2.67)	30.46 (2.34)
	2	37.65 (2.53)	30.74 (2.70)	38.03 (2.00)	31.29 (2.31)	37.58 (2.89)	30.30 (2.29)
	3	37.83 (2.28)	30.69 (2.64)	37.83 (1.84)	31.46 (2.92)	38.35 (2.43)	31.00 (2.75)
1	0.5	39.00 (2.10)	30.48 (2.35)	38.86 (1.94)	30.43 (1.29)	39.61 (2.75)	30.49 (2.12)
	1	38.94 (1.77)	30.85 (1.35)	38.95 (2.04)	30.90 (1.33)	38.95 (1.65)	30.74 (2.14)
	2	38.78 (1.36)	31.36 (1.57)	37.86 (2.30)	31.11 (1.34)	39.04 (1.91)	31.78 (1.77)
	3	38.51 (1.07)	31.30 (1.60)	38.74 (1.66)	31.25 (0.90)	38.69 (1.53)	31.41 (1.21)
1.5	0.5	39.06 (2.65)	30.20 (1.55)	40.54 (2.72)	30.20 (1.73)	40.61 (2.81)	30.83 (1.59)
	1	39.06 (2.80)	30.39 (1.29)	39.85 (2.47)	30.40 (2.07)	39.50 (2.28)	30.90 (1.59)
	2	38.91 (3.00)	30.73 (1.12)	39.13 (2.20)	30.95 (1.88)	39.85 (2.76)	30.96 (1.87)
	3	38.59 (2.48)	31.03 (1.25)	39.63 (2.24)	30.93 (2.41)	39.83 (2.53)	31.16 (2.00)
2	0.5	39.94 (3.42)	29.50 (2.48)	40.83 (3.69)	29.96 (2.65)	40.51 (2.89)	30.08 (1.79)
	1	40.36 (2.44)	30.50 (2.52)	40.14 (3.50)	30.18 (1.98)	39.85 (2.53)	30.40 (1.52)
	2	40.28 (3.27)	30.20 (2.60)	40.03 (3.52)	30.05 (2.61)	39.94 (3.35)	30.04 (2.39)
	3	39.69 (2.84)	30.14 (2.40)	39.69 (3.61)	29.94 (2.33)	39.15 (1.87)	30.51 (2.06)
2.5	0.5	40.69 (3.27)	29.51 (1.37)	40.44 (3.27)	29.20 (3.19)	41.26 (3.21)	30.64 (2.26)
	1	40.60 (3.50)	29.90 (1.04)	41.31 (3.23)	28.86 (2.14)	40.59 (2.92)	29.69 (1.73)
	2	40.05 (2.49)	29.88 (1.59)	39.90 (2.98)	29.81 (1.87)	41.06 (2.83)	30.26 (2.35)
	3	39.76 (2.44)	30.10 (1.83)	39.35 (3.05)	29.70 (1.80)	40.14 (2.22)	30.64 (1.47)

Table 3. Mean threshold values and standard deviations in parenthesis for Posture 3

Rate of temperature change: °C/s	Push force: N	Day 1		Day 2		Day 3	
		Warm threshold (°C)	Cold threshold (°C)	Warm threshold (°C)	Cold threshold (°C)	Warm threshold (°C)	Cold threshold (°C)
0.5	0.5	39.30 (2.80)	29.91 (2.85)	38.60 (3.09)	29.73 (2.64)	39.05 (2.51)	29.98 (2.45)
	1	38.64 (2.70)	30.23 (2.64)	38.54 (2.44)	29.61 (2.62)	38.14 (2.25)	29.94 (2.51)
	2	38.75 (2.47)	30.24 (2.32)	38.29 (2.82)	30.06 (2.97)	38.16 (2.04)	31.05 (2.47)
	3	38.43 (2.84)	30.45 (2.84)	38.01 (2.68)	30.71 (2.76)	38.03 (1.94)	30.15 (2.46)
1	0.5	39.34 (3.92)	29.15 (2.84)	39.68 (2.26)	29.39 (4.08)	39.64 (3.19)	30.21 (2.01)
	1	38.44 (2.60)	29.83 (2.78)	39.63 (3.02)	29.29 (3.00)	39.11 (2.57)	30.24 (2.40)
	2	39.26 (3.75)	29.55 (2.88)	39.56 (3.66)	30.58 (2.99)	38.60 (2.69)	30.45 (2.34)
	3	39.03 (3.46)	29.53 (2.44)	39.25 (3.37)	29.74 (3.22)	39.00 (2.85)	30.14 (2.45)
1.5	0.5	40.09 (3.41)	29.79 (2.49)	40.48 (3.85)	29.23 (3.54)	39.53 (3.19)	29.16 (2.69)
	1	39.88 (3.24)	30.35 (2.50)	39.55 (3.48)	29.28 (3.25)	38.81 (3.19)	29.50 (2.41)
	2	39.50 (2.52)	30.04 (2.54)	39.25 (3.34)	29.43 (2.93)	38.79 (2.99)	29.65 (2.75)
	3	39.65 (3.02)	29.91 (2.49)	39.55 (3.20)	29.86 (3.13)	38.60 (2.47)	29.39 (2.90)
2	0.5	40.80 (5.25)	29.08 (2.83)	41.66 (4.01)	29.38 (3.02)	41.14 (4.02)	29.39 (3.04)
	1	39.69 (4.40)	29.19 (2.28)	40.30 (4.14)	29.69 (3.40)	40.38 (4.07)	28.91 (3.29)
	2	39.79 (3.78)	29.80 (2.22)	40.56 (3.10)	29.99 (3.07)	40.29 (3.88)	30.20 (3.15)
	3	39.34 (3.55)	28.73 (3.52)	40.29 (3.66)	29.98 (2.80)	40.04 (3.16)	29.44 (3.07)
2.5	0.5	41.38 (4.62)	28.98 (2.58)	41.24 (3.31)	29.41 (3.02)	42.09 (3.76)	29.03 (3.12)
	1	41.10 (4.66)	29.23 (2.21)	41.83 (3.68)	29.08 (2.75)	41.83 (4.30)	29.38 (2.88)
	2	41.08 (4.64)	29.38 (2.77)	41.48 (4.40)	29.50 (2.82)	40.80 (3.02)	29.24 (3.47)
	3	40.70 (4.16)	29.85 (2.11)	40.34 (3.33)	29.93 (2.78)	40.58 (3.52)	29.84 (2.91)

Table 4. Differences in thermotactile perception threshold among measurement conditions

Rate of change of applicator temperature	Push force	Posture	Measurement day		
			Day 1	Day 2	Day 3
Cold threshold					
1°C	0.5 N	Posture 1	32.0 ± 1.3	31.9 ± 1.7	31.8 ± 1.4
		Posture 2	30.5 ± 2.3	30.4 ± 1.3	30.5 ± 2.1
		Posture 3	29.2 ± 2.8^{+}	29.4 ± 4.1	30.2 ± 2.0
1°C	0.5 N	Posture 1	32.0 ± 1.3	31.9 ± 1.7	31.8 ± 1.4
	1.0 N		32.4 ± 1.8	32.4 ± 1.8	32.4 ± 1.0*
	2.0 N		32.3 ± 1.0	32.1 ± 1.3	31.5 ± 1.7
	3.0 N		32.6 ± 1.0*	32.3 ± 1.5	32.3 ± 1.8
0.5°C	0.5 N	Posture 1	31.7 ± 1.0	31.1 ± 1.7	31.4 ± 1.7
1.0			32.0 ± 1.3	31.9 ± 1.7	31.8 ± 1.4
1.5			31.3 ± 0.9	30.9 ± 1.0	30.8 ± 2.0
2.0			30.4 ± 1.3*	30.4 ± 1.7	30.3 ± 1.7
2.5			31.4 ± 1.7	31.1 ± 1.5	31.5 ± 0.9
Warm thresholds					
1°C	0.5 N	Posture 1	38.7 ± 2.0	38.0 ± 1.9	37.8 ± 1.6
		Posture 2	39.0 ± 2.1	38.9 ± 1.9	39.6 ± 2.8^{+}
		Posture 3	39.3 ± 3.9	39.7 ± 2.3*	39.6 ± 3.2
1°C	0.5 N	Posture 1	38.7 ± 2.0	38.0 ± 1.9	37.8 ± 1.6
	1.0 N		37.9 ± 2.3	38.3 ± 1.9	38.4 ± 1.4
	2.0 N		38.4 ± 1.9	37.9 ± 1.9	37.5 ± 2.2
	3.0 N		37.9 ± 1.4*	37.9 ± 1.7	37.9 ± 1.6
0.5°C	0.5 N	Posture 1	37.5 ± 1.6	37.6 ± 1.9	38.5 ± 1.9
1.0			38.7 ± 2.0	38.0 ± 1.9	37.8 ± 1.6
1.5			38.3 ± 1.6	39.3 ± 2.4	39.0 ± 2.3
2.0			39.7 ± 2.4	40.3 ± 1.9^{+}	40.4 ± 2.0*
2.5			40.4 ± 1.8*	39.7 ± 2.0	40.4 ± 1.5*

$^{+}p<0.1$, *$p<0.05$.

slightly.

Repeated measures ANOVA showed that the warm threshold was significantly affected by the rate of temperature change ($F=3.449$, $p<0.05$) and the push forces ($F=27.816$, $p<0.01$). The cold threshold was significantly affected by the push forces ($F=9.725$, $p<0.01$), posture ($F=9.998$, $p<0.01$) and the interactions between push forces and the posture ($F=3.651$, $p<0.05$). The effect of measurement day was not significant ($F=1.173$, $p=0.338$ for warm thresholds; $F=0.206$, $p=0.817$ for cold thresholds), so that factors of posture, push force and rate of temperature change affected warm or cold threshold measurements.

Differences among measuring conditions for posture, rate of temperature change, and push force were then compared by means of paired t-test. Some of the results are shown in Table 4.

Under the measuring conditions with a 0.5 N push force at a change rate of 1°C, statistical differences in cold or warm thresholds were not encountered among different measuring postures. Nevertheless, warm thresholds tended to increase in the order posture 1, posture 2 and posture 3, and the differences between posture 1 and posture 3 were on average 1.6–2.5°C for cold thresholds and 0.6–1.8°C for warm thresholds. On the other hand, although significant differences in thermal thresholds were observed among different conditions of push force, the difference was less than 1°C: 0.5–0.9°C for cold thresholds and 0.4–0.8°C for warm thresholds. Statistical differences were found among different rates of change in temperature, particularly in warm thresholds. The differences were 0.6–1.5°C for cold thresholds and 2.6–2.9°C for warm thresholds. It is therefore thought that it is necessary to standardize the measuring posture and the rate of temperature change as fundamental measurement conditions, whereas the effect of the push force may be small in practice.

Table 5. Mean values for subject comfort in thermotactile measurement

Rate of temperature change: °C	Push force: N	Posture 1	Posture 2	Posture 3
0.5	0.5	0.6	0.4	–0.9
0.5	1	0.6	0.3	–0.9
0.5	2	0.7	0.3	–1
0.5	3	0.6	0.3	–1
1	0.5	0.7	0.5	–0.8
1	1	0.7	0.5	–0.8
1	2	0.6	0.4	–0.9
1	3	0.6	0.3	–0.9
1.5	0.5	0.7	0.5	–0.8
1.5	1	0.7	0.5	–0.8
1.5	2	0.6	0.4	–0.9
1.5	3	0.5	0.4	–0.9
2	0.5	0.7	0.5	–0.7
2	1	0.7	0.5	–0.7
2	2	0.6	0.5	–0.8
2	3	0.5	0.5	–0.8
2.5	0.5	0.7	0.5	–0.7
2.5	1	0.7	0.5	–0.7
2.5	2	0.6	0.5	–0.8
2.5	3	0.6	0.5	–0.8

Subject's comfort assessment of measuring condition

Table 5 shows the mean values for subject comfort assessment of each measurement condition.

A value close to 1 showed that the measuring condition was comfortable for the subject. Posture 3, with straight hand and forearm without a support was the most uncomfortable posture, whereas posture 1 with straight hand and forearm with a support was the most comfortable for the subjects. The values for the comfort assessment were likely to increase a little with a decrease in the push force. The rate of temperature change seemed to have only a little effect.

Repeatability of threshold measurement

The repeatability of thermal measurement on different days was evaluated with correlation coefficients and r.m.s. errors. Good repeatability should show a high correlation coefficient and a small r.m.s. error[8]. The correlation coefficients varied among different measuring postures. The r.m.s. errors tended to be greater in posture 2, but the errors were almost the same in postures 1 and 3. Nevertheless, the comfort assessment showed that posture 1 was the most comfortable for the subjects. Table 6 shows Pearson's correlation coefficients and r.m.s. errors for different measurement days for posture 1 only.

The correlation coefficients for both warm and cold thresholds on different days under the same measurement conditions were relatively high for the following four conditions: a 0.5°C/s temperature change rate and a 2 N push force, a 1°C/s temperature change rate and a 0.5 N push force, a 1.5°C/s temperature change rate and a 1 N push force, and a 2°C/s temperature change rate and a 0.5 N push force.

Among these four conditions, the r.m.s. errors looked the smallest under the measurement condition of a 1°C/s temperature change rate and a 0.5 N push force.

Discussion

In the present study, the measurement postures significantly affected cold threshold measurements. The means and standard deviations of thermal thresholds were likely to be smallest in posture 1 (straight hand and forearm on a support), and they tended to be greatest in posture 3 (straight hand and forearm without a support). In posture 2 (the fingers and hand flexed at the wrist with the elbow placed on a desk) they were in between them. These results may be associated with the stability of the hand and arm examined. In posture 2, the flexion or rotation of the wrist during the measurements might compress the median or ulnar nerve

Table 6. Pearson correlation coefficients and root-mean-square differences in parenthesis for cold and warm thresholds obtained in Posture 1 on different days

Rate of temperature change: °C/s	Push force	Cold thresholds			Warm thresholds		
		Days 1 and 2	Days 1 and 3	Days 2 and 3	Days 1 and 2	Days 1 and 3	Days 2 and 3
0.5	0.5	0.604 (4.162)	0.521 (4.096)	0.699 (3.564)	0.926** (1.980)	0.841** (3.838)	0.850** (3.783)
	1	0.542 (4.893)	0.592 (4.053)	0.865** (2.751)	0.635 (3.548)	0.721* (3.620)	0.741* (3.724)
	2	0.831* (2.179)	0.846** (2.392)	0.788* (2.830)	0.910** (2.804)	0.931** (3.046)	0.759* (4.427)
	3	0.749* (3.932)	0.481 (4.720)	0.871** (2.909)	0.780* (2.998)	0.769* (3.582)	0.631 (3.878)
1	0.5	0.668 (3.330)	0.841** (2.074)	0.826* (2.496)	0.879** (3.208)	0.847** (3.775)	0.939** (1.849)
	1	0.734* (3.497)	0.383(4.587)	0.383 (4.419)	0.340 (6.702)	0.439 (5.849)	0.819* (2.998)
	2	0.269 (3.822)	–0.045 (5.762)	0.486 (4.458)	0.700 (4.190)	0.534 (5.980)	0.905**(2.786)
	3	0.695 (3.082)	0.520 (4.120)	0.440 (4.647)	0.919** (1.844)	0.628 (3.514)	0.832* (2.571)
1.5	0.5	0.114 (3.576)	0.490 (4.761)	0.440 (4.732)	0.519 (6.304)	0.482 (9.880)	0.728* (4.741)
	1	0.715* (2.839)	0.719* (3.197)	0.845* (2.163)	0.720* (3.837)	0.529 (4.490)	0.785* (3.493)
	2	0.690 (1.775)	0.778* (3.309)	0.598 (3.493)	0.716* (2.968)	0.826* (2.702)	0.798* (2.617)
	3	0.164 (3.947)	0.404 (3.212)	0.652 (2.135)	0.656 (2.938)	0.576 (2.702)	0.384 (3.956)
2	0.5	0.868** (2.332)	0.742* (3.033)	0.770* (3.105)	0.754* (4.500)	0.919** (3.305)	0.872** (2.631)
	1	0.523 (3.481)	0.592 (3.611)	0.714* (3.243)	0.926** (3.089)	0.823* (3.828)	0.927** (2.211)
	2	0.233 (4.595)	–0.093 (4.903)	0.647 (4.134)	0.829* (3.504)	0.848** (2.825)	0.788* (3.295)
	3	0.216 (4.227)	0.149(4.312)	0.862** (1.975)	0.817* (4.009)	0.816* (3.263)	0.758* (4.660)
2.5	0.5	0.581 (3.989)	0.782* (2.968)	0.516 (3.578)	0.864** (3.457)	0.805* (2.850)	0.919** (3.158)
	1	0.407 (4.855)	0.744* (3.061)	0.711* (3.252)	–0.045 (4.387)	0.357 (3.966)	–0.351 (3.960)
	2	0.536 (2.968)	0.745* (2.492)	0.739 (2.417)	0.804* (2.850)	0.277 (8.471)	–0.067 (10.10)
	3	0.677 (3.298)	0.760* (1.709)	0.623 (3.418)	0.043 (5.378)	0.511 (3.477)	0.560 (2.944)

*p<0.05; **p<0.01.

and affect the threshold[6]. The present findings on subject's comfort assessment also indicated that the posture 1 was the most comfortable for the subjects, and posture 3 was the reverse, so that the posture in which the straight hand and forearm are laid on a support can be the most comfortable for the subjects and is recommended for thermal threshold measurements.

The present repeated ANOVA also indicated that the rate of change in the applicator temperature significantly influenced warm thresholds, and the push force affected warm and cold thresholds. As the rate of temperature change rose from 0.5°C/s to 2.5°C/s, warm thresholds tended to increase. Hilz *et al.* also showed that thermal thresholds were higher with a 3°C/s stimulation than with a 1°C/s stimulation[3]. Ruffell and Griffin found that incremental rates of temperature did not significantly affect thermal thresholds, except that cold thresholds were significantly higher at 0.5°C/s than at 2.5°C/s[10]. On the other hand, increasing the push force tended to produce slightly lower thermal thresholds. Although there were no studies investigating the effect of the push force on thermal thresholds, similar findings were shown by previous studies on vibrotactile thresholds[5, 9].

When the repeatability of thermal thresholds was compared in the measuring posture of the straight hand and forearm on a support, a relatively good repeatability was obtained at a 1°C/s temperature change rate and a 0.5 N push force for warm and cold thresholds. The repeatability was evaluated with correlation coefficients and r.m.s. errors[8]. Relatively good repeatability was observed in various measuring conditions for warm thresholds, but for cold thresholds it was encountered in a small number of measuring conditions, as shown in Table 6, where repeatability differed with the rate of temperature change and the push force. Considering both warm and cold thresholds, the present findings have shown that a 1°C/s temperature change rate and a 0.5 N push force are recommendable measuring conditions. A 1°C/s temperature change rate was in accordance with previous studies[3, 6, 10], where the effect of the temperature change rate was investigated with a fixed push force of 2 N. In thermal threshold measurements, a 2 N push force was often used[6, 10], but for Japanese subjects, a weaker push force may be more suitable, since similar findings were found in vibrotactile studies[9]. In comparison of 0.5°C/s and 1°C/s temperature change rates, 1.0°C/s is

also preferable to 0.5°C/s in practice, because the former condition requires only a shorter measurement time than the latter. And it turns out that the shorter measurement time is similar to that shown in Table 4. In view of this, if measurement time becomes long in actual on-site measurement, it will be expected that the subject is made to feel unpleasant.

In the present study the starting temperature of the applicator was set at the subject's finger skin temperature. Some studies have shown that when the skin temperature of the subject is over 30°C, thermal testing is sufficiently accurate[3, 11]. The present experiments were conducted under conditions in which the skin temperature of all subjects was over 30°C (34.4 ± 1.2°C) in a soundproof room with an air temperature of 24 to 26°C. It is therefore considered that the effect of the starting temperature is negligible.

Conclusion

The present series of experiments investigated the effect of measurement postures, the rate of change in temperature, and push forces on thermotactile thresholds, by means of an *HVLab* thermal aesthesiometer. Warm thresholds were significantly affected by the push force and the rate of temperature change and cold thresholds were influenced by postures and push force. The measurement posture of straight hand and forearm laid on a support was the most comfortable for the subjects. A push force of 0.5 N and a rate of temperature change of 1.0°C/sec in posture 1, straight hand and forearm laid on a support can be suitable conditions for the measurement of warm and cold thresholds from the viewpoints of repeatability of measurement and subject's comfortable.

Acknowledgement

This study was supported by a grant-in-aid for scientific research (no. 09670385) from the Ministry of Education, Science, Sports and Culture of Japan. This study was performed at the Human Factors Research Unit, Department of Industrial Engineering, Faculty of Science and Technology, Kinki University, Higashi-Osaka, 577-8502, Japan. The authors would like to acknowledge the students of the Human Factors Research Unit, Kinki University for their valuable assistance in this series of experiments.

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Industrial Health 2002, **40**, 362–369 **Original Article**

Effects of pH and Temperature on Force and Stiffness of Skeletal Muscle Fibers during Contraction and Relaxation in Relation to Musculoskeletal Disorders

Satoru UENO[1, 2]*, Kazuhito YOKOYAMA[2], Michinori NAKAGAWA[1] and Shunichi ARAKI[1]

[1] National Institute of Industrial Health, 6-21-1,Nagao, Tama-ku, Kawasaki 214-8585, Japan

[2] Department of Public Health, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

Received February 1, 2002 and accepted August 28, 2002

Abstract: Effects of low pH (6.0,6.5) were studied at three temperatures (5, 15, 25°C) on the isometric force and stiffness of glycerinated muscle fibers dissected from soleus (type I) and psoas (type IIX) muscles of the Japanese white rabbit. It was observed that the maximum force and stiffness declined as pH decreased, the extent of which was diminished by an increase in temperature in both muscle types. The pH-induced changes in force were greater than those in the stiffness for both muscle types: at 5°C, the psoas showed greater change in stiffness than the soleus. As the pH of the contracting solution decreased, the time to peak stiffness decreased and the time to relaxation increased in both muscle types. At pH 6.0, the latter was significantly longer than the former. The force-stiffness curves indicated that, when pH decreased, force increased as fast as stiffness during contraction and force declined faster than stiffness during relaxation. The results suggested that one of the causes of muscle injury during hard muscular work could be the longer relaxation time in an acidic solution.

Key words: Skeletal muscle, Force, Stiffness, pH, Temperature

Introduction

Musculoskeletal disorders account for the majority of occupational diseases[1, 2]. The Japanese Ministry of Labor reported in 1999 that in cases of occupational sick leave for 4 days or more, over 50% involved musculoskeletal disorders[3]. Such disorders tend to happen after strenuous unaccustomed exercise or repetitive motion[4]. In muscle fatigue, the pH decreases as lactate and inorganic phosphate increase in the muscle, and muscle force declines. Data from human first dorsal interosseous muscle with 31P-MRS[5] indicated that pH decreased from about 7.0 to 6.0 after exhausted contraction. In order to understand the mechanism of muskuloskeletal injury, it is important to investigate the muscle force and stiffness of various muscle types in various pH conditions. In this study, we investigated the contractile characteristics of soleus (type I, slow) and psoas (type IIX, fast)[6] muscles of the Japanese white rabbit at low pH at three different temperatures.

*To whom correspondence should be addressed.

Materials and Methods

Skeletal muscles

Female rabbits (Japanese White Rabbit), aged from 8 to 10 weeks, were sacrificed by rapid neck disarticulation. Fiber bundles (1–2 mm in diameter and 5–6 cm in length) were dissected from the psoas and soleus muscles, slightly stretched and tied to glass capillary tubes, left to stand for half an hour in a solution containing 154 mM NaCl, 5.6 mM KCl, 2.4 mM $NaHCO_3$ and 0.5% Triton X-100 at 4°C, and then stored in a solution of 50% glycerol, 50 mM KCl, 4 mM $MgCl_2$, 4 mM K_2EGTA, and 20 mM Tris(hydroxymethyl) aminomethane-maleic acid (pH 7.0) at –20°C for up to 14 days. On the day of the experiment,

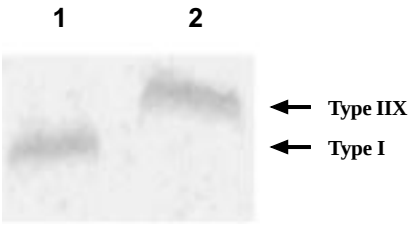

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of myosin heavy chain isoforms from rabbit psoas and soleus muscles.

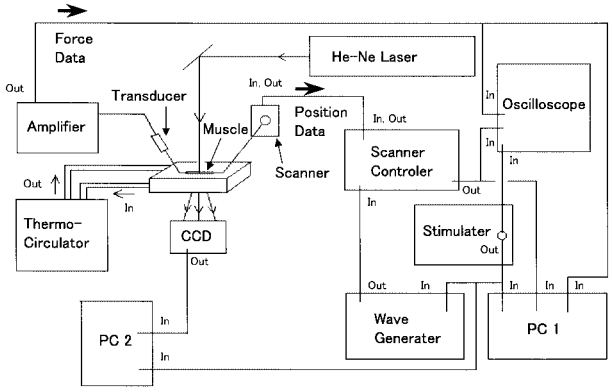

Fig. 2. A schematic diagram of the experiment apparatus. Muscle force was detected with a transducer, amplified, and stored in PC1. The muscle was pulled by a tungsten-steel pin attached to a scanner, the position of the pin being controlled by a signal from a wave generator. Sarcomere length was measured by laser diffraction.

a glycerinated fiber was isolated from the bundle under the optical microscope with sharp-pointed tweezers in a solution of the same composition for storage at 0°C and mounted horizontally in the experimental chamber.

Fiber type composition

The myosin heavy chain (MHC) isoforms of some single fibres were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a the separating solution containing 5% (w/v) polyacrylamide, 40% (v/v) glycerol, 0.1% (w/v) SDS, 0.4 M Tris(hydroxymethl)aminomethane (Tris)-HCl (pH 8.8), 3% (w/v) $(NH_4)_2S_2O_8$, 0.5‰ (v/v) N,N,N',N'-Tetramethylethylenediamine (TEMED) and the stacking solution containing 3.5% (w/v) polyacrylamide, 40% (v/v) glycerol, 0.1% (w/v) SDS, 0.13 M Tris-HCl(pH 6.8), 3‰ (w/v) $(NH_4)_2S_2O_8$, 0.5‰ (v/v) TEMED. Gels were stained in a solution containing 0.25% (w/v) Coomassie brilliant blue, 45% (v/v) ethanol, and 10% (v/v) acetic acid and destained in a solution containing ethanol and acetic acid. As shown in Fig. 1, MHC compositions were type I and type IIX for soleus and psoas muscles respectively, indicating that fast and slow muscle types did not mingle.

Experimental apparatus

The experimental apparatus is shown schematically in Fig. 2. Both ends of the single skinned fiber were glued to tungsten-steel rods with collodion diluted 1:1.5 in ethanol, one being connected to a force transducer (801, Akers, Horten, Norway) and the other connected to a light arm from a rapid motor (General Scanning Inc, Watertown, MA) to change muscle length. The force transducer (resonance frequency 10 ± 0.5 kHz) was connected mechanically to a three dimensional manipulator and electrically to a force bridge amplifier. The rapid motor was connected to a scanner controller (CX-660, General Scanning Inc, Watertown, MA) to which positional data were sent from a wave generator (Wave Factory 1945, NF Electronic Instruments).

The sarcomere length of the single skinned fiber under relaxed conditions was measured by laser diffractometry (He-Ne laser, 632.8 nm, 5 mW, NEC) and adjusted to 2.4 μm[7, 8], as force and stiffness differed according to an overlap of the thick and thin filaments[9]. The signals from the force transducer and scanner controller were amplified by a DC amplifier (model 6L02, NEC) and stored at 10 kS/sec in a personal computer (PC1) through an AD-DA converter board (AT-MIO-16E-10, National Instruments), which digitized records with 12-bit resolution. Software (Labview 5.1, National Instruments) was used for data acquisition and experimental control. The data were stored for ten single fibers, each of which was measured at least three times.

Two troughs to which solution was added were constructed in an aluminum block to facilitate fiber handling and solution exchange. One trough was filled with relaxing solution and the other with contracting solution. The solution in which the muscle fiber was soaked was changed by shifting the aluminum block and suspending the muscle fiber in air for a brief time. During the experiments, the temperature of the aluminum block supporting the experimental solution was monitored with a thermometer and the block temperature drift was controlled within 0.3°C by passing coolant from a thermo-circulator (ZL-100, Taitec, Japan) through the block.

Solutions

The relaxing solution consisted of 125 mM KCl, 20 mM PIPES, 4 mM ATP, 4 mM $MgCl_2$, and 4 mM EGTA. The contracting solution was obtained by adding 4 mM $CaCl_2$ to the relaxing solution. The pH of the relaxing and

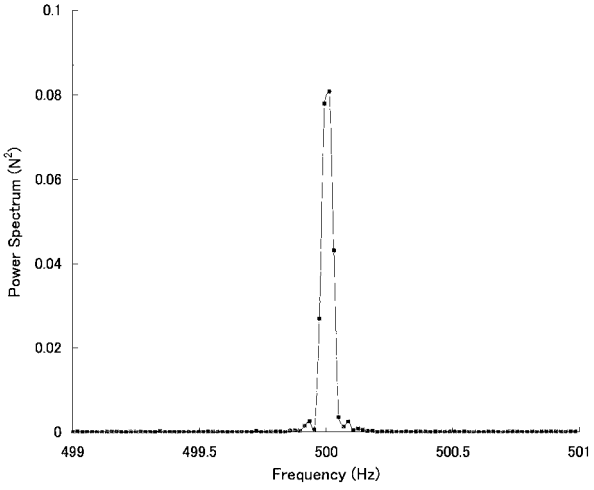

Fig. 3. A power spectrum for the force of psoas muscle around 500 Hz.

contracting solutions was adjusted to 6.0, 6.5 and 7.0 by adding HCl or KOH solutions. In this study, the amount of ATP in solution was so large that the percentage of hydrolyzed ATP was negligible.

Measurement of muscle stiffness

Muscle fiber stiffness was calculated from the recorded force data. Fiber stiffness was measured by the change in force that was caused by a fast, low-amplitude length change in the muscle fiber[10, 11]. A 500 Hz sinusoidal length oscillation whose amplitude was 0.1% the length of the muscle fiber was applied to one end of the fiber, during which force response was measured at the other end. Although the frequency in our study was lower than in other studies[11], and could thus result in a lower absolute stiffness value, it did not present much of a problem because in our analysis we used relative stiffness. The stored data were analyzed by Fast Fourier Transform (FFT) spectral approaches.

Fig. 3 is a typical example of a Fourier spectrogram for force of the psoas muscle around 500 Hz. Since the width of the peak was within 0.4 Hz, we set the width of the band pass filter to 1 Hz to extract 500 Hz components from muscle force data disturbed by the length change. The extracted components in the Fourier space were returned to real space by Inverse FFT (IFFT). The wave width of 500 Hz components in real space was divided by the width of the length oscillation to arrive at the muscle stiffness. Fig. 4 shows the time course of the isometric force and stiffness of a single glycerinated rabbit psoas fiber at pH 7.0, 15°C. Since the change in muscle temperature brought changes in the time course of stiffness, the room temperature was kept

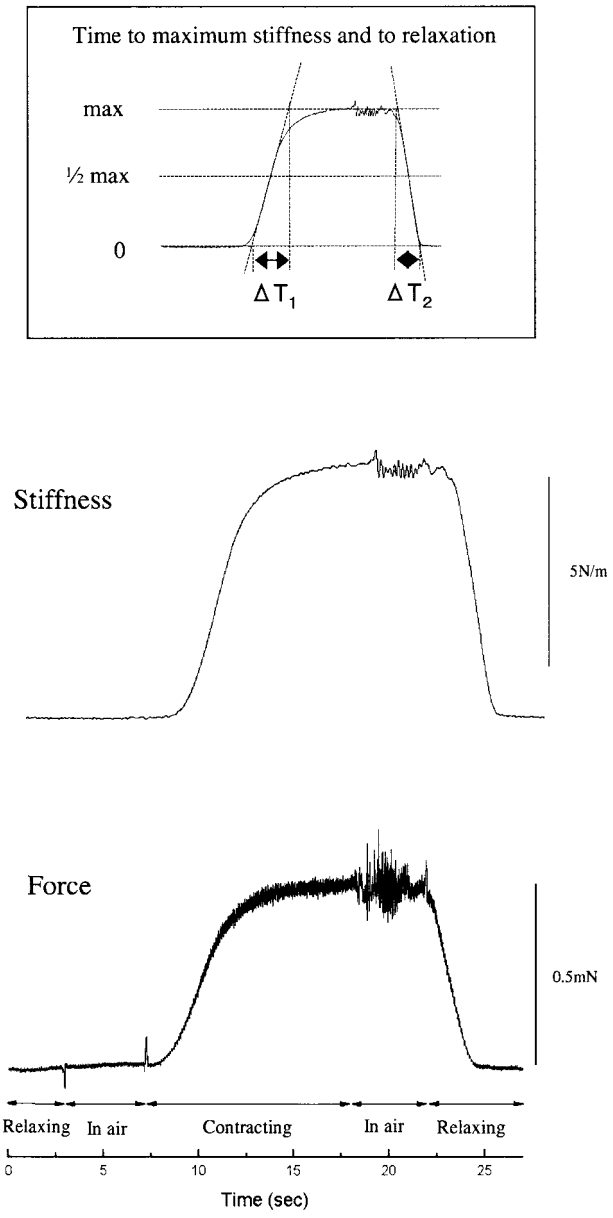

Fig. 4. Force and stiffness measured in a single psoas muscle fiber. The muscle fiber was first immersed in a Ca^{2+} free relaxing solution, and then in a Ca^{2+} contracting solution. Finally it was returned to the Ca^{2+} free relaxing solution. Insert: Estimation of time to maximum stiffness (ΔT_1) and to relaxation (ΔT_2) on stiffness curve.

the same as the solution temperature.

Time to maximum stiffness and relaxation

As shown in the insert in Fig. 4, time to maximum stiffness and relaxation was estimated by dividing maximum stiffness by increasing and decreasing the speed of stiffness at half maximum stiffness.

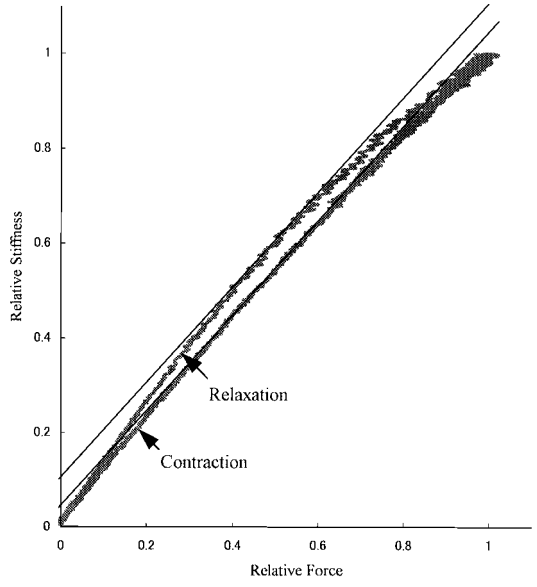

Fig. 5. Force-stiffness curve during contraction and relaxation of a single psoas muscle fiber in pH 6.0 at 15°C. Force and stiffness of the muscle fiber reached maximum at the end of contraction. Then the muscle was soaked in the relaxing solution, resulting in decreases in force and stiffness. A tangential line was drawn to estimate the curvature of the contraction and relaxation curves.

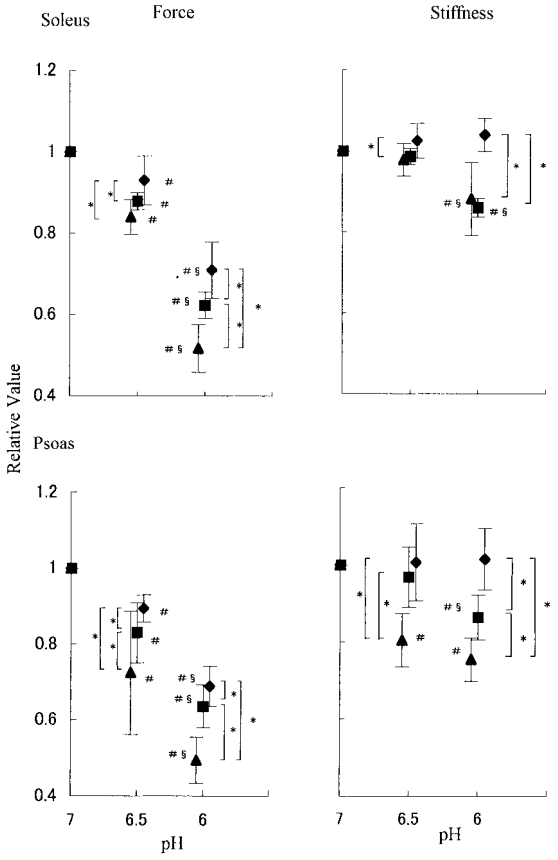

Fig. 6. Changes in maximum force and stiffness of soleus and psoas muscles concomitant with the decrease in pH. The values are adjusted to 1.0 at pH 7.0. ▲, ■, ◆ = at 5, 15 and 25°C, respectively (n = 10 for each). * = $p<0.05$. # = $p<0.05$ (compared with at pH 7.0). § = $p<0.05$ (compared with at pH 6.5).

Force-stiffness curve

To analyze force-stiffness relationships during muscle relaxation and contraction, the relative stiffness was plotted against the relative force under each pH. As exemplified in Fig. 5, the force-stiffness curve is convex; the Y intercept of the tangenital line, which is parallel to X=Y, to the curve was calculated to express the degree of curvature. The larger the Y intercept is, the more the curve is detached upward from the X=Y line, indicating an enlarging of difference of increasing or decreasing speed between relative force and stiffness. The curvature of force-stiffness curve of a psoas muscle fiber in relaxation process was larger than that in contraction process at pH 6.0 and 15°C.

Experimental schedule

When the effects of pH on muscle force and stiffness were examined, the temperatures of the contracting and relaxing solutions were fixed at 5, 15 or 25°C. Measurements were made for a single muscle fiber, soaked in relaxing and contracting solutions alternately at the same pH (Fig. 4), and this cycle was repeated for different pHs, i.e. 6.0, 6.5, 7.0, 7.0, 6.5, 6.0, 6.0, 6.5 and 7.0, in that order. If the last maximum force at pH 6.0 was larger than 70% of the first pH 6.0, the data were used for analysis. The room temperature was kept at the same temperature as that of the solution so that muscle temperature did not change when changing the solution.

Statistics

Effects of pH, temperature and muscle type on relative force and stiffness were analyzed with SAS/STAT software ver. 6.11 (SAS Institute Inc.) run on an S-4/5 computer (Fujitsu).

Results

PH effects on the relative maximum force and stiffness of the soleus and psoas muscle for each temperatures are shown in Fig. 6 and Table1. There were three effects caused by pH changes. The first was that both maximum force and stiffness declined significantly in an acidic solution. For example, maximum force of psoas muscle decreased by 27.5% (pH

Table 1. Differences between soleus and psoas muscle fibers in maximum force and stiffness (mean ± standard deviation)

			Soleus (n=10)	Psoas (n=10)	P
Force	PH 6.5	25°C	0.930 ± 0.060	0.894 ± 0.036	0.021
		15°C	0.879 ± 0.021	0.830 ± 0.079	0.011
		5°C	0.840 ± 0.043	0.725 ± 0.163	0.001
	PH 6.0	25°C	0.710 ± 0.070	0.689 ± 0.053	0.468
		15°C	0.623 ± 0.032	0.636 ± 0.057	0.574
		5°C	0.517 ± 0.059	0.494 ± 0.061	0.426
Stiffness	PH 6.5	25°C	1.026 ± 0.043	1.007 ± 0.100	0.301
		15°C	0.987 ± 0.020	0.969 ± 0.078	0.605
		5°C	0.979 ± 0.040	0.805 ± 0.069	0.000
	PH 6.0	25°C	1.040 ± 0.041	1.015 ± 0.080	0.129
		15°C	0.860 ± 0.023	0.864 ± 0.058	0.903
		5°C	0.882 ± 0.090	0.755 ± 0.056	0.008

Adjusted to 1.0 at pH 7.0.

Table 2. The time from relaxation to maximum stiffness and time from peak to relaxation

		Time to Maximum Stiffness (sec)			Time to Relaxation (sec)		
	pH	7	6.5	6	7	6.5	6
Soleus	25°C	2.06 ± 0.45	1.46 ± 0.54	1.26 ± 0.31	2.75 ± 0.62*	4.06 ± 0.97*§	6.76 ± 1.67*§
	15°C	9.37 ± 2.76	4.64 ± 0.91	2.83 ± 0.37	2.78 ± 0.49	6.30 ± 1.06*§	10.86 ± 2.22*§
	5°C	28.37 ± 4.18	17.36 ± 5.14	6.81 ± 1.38	4.73 ± 1.69	7.39 ± 2.01§	27.85 ± 5.32*§
Psoas	25°C	3.19 ± 0.59	2.16 ± 0.90	1.89 ± 0.84	1.66 ± 0.27	2.57 ± 0.57§	3.85 ± 1.19*§
	15°C	3.12 ± 0.81	1.80 ± 0.77	1.56 ± 0.71	2.57 ± 0.21	4.77 ± 0.98*	9.08 ± 1.92*§
	5°C	9.11 ± 2.40	5.03 ± 1.22	3.39 ± 0.66	2.90 ± 0.63	6.50 ± 1.72*	11.27 ± 2.39*§

§ = $p<0.05$, when time to relaxation is significantly longer than time to maximum stiffness of the another kind of muscle.
* = $p<0.05$, when time to relaxation is significantly longer than time to maximum stiffness of the same kind of muscle.

6.5) and 50.6% (pH 6.0) at 5°C and maximum stiffness decreased by 19.5% (pH 6.5) and 24.5% (pH 6.0) at 5°C. The second was that the pH dependent decline in force and stiffness was significantly depressed by raising the temperature, e.g. The forces of psoas muscle at pH 6.0 were 31.1% (25°C), 36.4% (15°C) and 50.6% (5°C) of that at pH 7.0, respectively. The third was that the reduction in muscle stiffness brought about by lowering the pH was less than that for muscle force.

The relative force was significantly smaller in psoas muscle than in soleus muscle at 25°C, 15°C and 5°C for pH 6.5. Similarly, relative stiffness was significantly less in the psoas muscle than in the soleus muscle at 5°C for pH 6.5 and 6.0.

Time to maximum stiffness and to relaxation of psoas and soleus muscle are shown in Table 2 for different pHs and temperature. As temperature decreased, both the time to maximum stiffness and to relaxation increased. As pH decreased, the time to maximum stiffness became longer and the time to relaxation became shorter. At pH 6.0, the time to maximum stiffness was significantly shorter than the time to relaxation in both fast and slow muscle fibers for all three temperatures.

Indices of the curvature of the force-stiffness curve (Fig. 7) of muscle fibers are shown in Fig. 8. During contraction, the curve in the acidic solution was less convex than in the neutral solution, i.e. the increase in the force was as fast as for the stiffness. In contrast, during relaxation, the curve in the acidic solution was more convex than in the neutral solution, i.e. the stiffness decreased more slowly than the force.

Discussion

Reduction in maximum force due to low pH became smaller at higher temperature, the mechanism for which is unknown[12]. It was demonstrated by using X-ray diffraction that the actin-myosin complex of a single permeabilized muscle fiber was transformed from a nonstereo-specific to

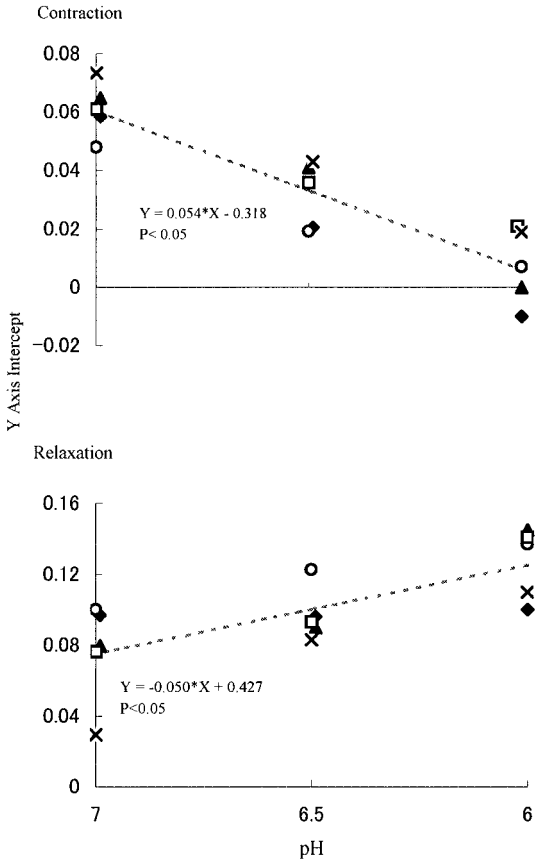

Fig. 7. Y-axis intercept of tangential line to the force-stiffness curve during contraction and relaxation at 15°C.
Five single psoas fibers (○, ▲, ×, □, ◆) were analyzed.

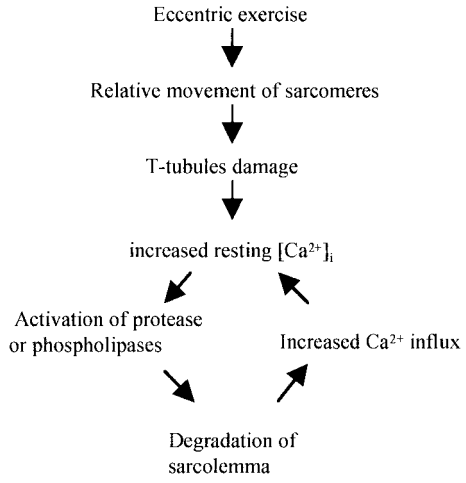

Fig. 8. Supposed mechanisim[17, 18] of muscle damage caused by eccentric exercise.

a stereo-specific structure at high temperature[13]. This suggested that the actin-myosin binding was strengthened by high temperature, resulting in an increase in muscle tension.

In the present study the maximum force of fast muscle fibers decreased with a lower pH more than in slow muscle. Metzger and Moss[14] found, in a single skeletal muscle fiber experiment, that as the pH decreased from 7.0 to 6.5, the maximum force decreased to 0.80 and 0.75 of its value at pH 7.0 for slow and fast muscles, respectively. Potma *et al.*[15] observed also that force decreased more in fast muscle than in slow muscle as the pH decreased from 7.9 to 6.4. As slow muscle fiber contains a higher mitochondrial content[16], amount of oxidative enzyme and blood vessel density, these finding suggest that slow muscles generate force even under the acidic conditions caused by strenuous exercise. Thus in a normal muscle which contains both fast and slow fibers, some stress between myofibrils could occur.

A cause of muscle damage suggested by the results of the present study was the shorter time to maximum stiffness during contraction and the longer time to relaxation under low pH conditions (Table 2). This suggests that the relaxing muscle which still contains force would be stretched forcibly if time to maximum force of its antagonist muscle is shorter than the time to relaxation of relaxing muscle. Time to maximum force could be considered to be close to time to maximum stiffness at low pH from the force-stiffness relationship (Fig. 8). Conversely, when the relaxed muscle contracts, the other side muscle which had contracted could be stretched. Then in repetitive work, both antagonist muscles could be stretched alternately. This stretch could lead to sarcomere length instability, T-system disrupture, Ca^{2+}, Na^{+} or pH regulation failure in muscle cells, protease activation and cellular component degradation, causing Ca^{2+} leakage and self-accelerating muscle damage[17, 18] (Fig. 9). Damaged muscle cells release chemical substances such as bradykinin and histamines which cause pain. This could be one of the causes of low back pain which often occurs during heavy work.

At low pH, force increased as fast as stiffness during contraction but force decreased faster than stiffness during relaxation. This could be explained in term of an ATPase cycle scheme[19]. At first, myosin attaches actin (weakly-binding state). Then the two molecules bind strongly (strongly-binding state) exerting a contracting force. Finally, actin and myosin detach. There is a latency between the force exerting state and the detachment, when force is not generated, but the muscle fiber produces stiffness during this latency, i.e. stiffness comes from the attachment of myosin to actin and disappears when they detach. It could therefore be supposed that at low pH the transition from the

weakly-binding state to the strongly-binding state progresses rapidly since the force increases as fast as the stiffness. Also, at lower pH, the latency between the force-exerting and actin-myosin detachment becomes longer, since force decreases faster than stiffness. These assumptions could explain the observation that the time to maximum stiffness is shorter and the time to relaxation is longer at low pH. Furthermore, they could explain why stiffness does not decrease as much as force at low pH or temperature, because it could be assumed that there are many actin and myosin attachments which produce muscle force without force generation during longer latency.

Low pH affects not only the interaction of actin and myosin but also other chemical interactions. It was reported that pH reduction affects control proteins on the actin filament by lowering Ca^{2+} sensitivity[20]. As the contracting solution contained an extra amount of Ca^{2+} in the present study, we did not have to consider low Ca^{2+} sensitivity. *In vivo*, since muscle cells did not contain extra Ca^{2+}, reducing Ca^{2+} sensitivity by lowering pH might lead to a longer contraction time or a reduction in force.

In this study, rabbit skeletal muscle was used in place of human muscle for the following reasons: (1) Concerning actin, the amino acid arrangement is saved in the process of heredity, e.g. mammalian, amoebae and yeast actins have more than 87% homology with each other[21]. (2) In myosin heavy chain, it is reported that the degree of homology of cording regions between the same isoforms in different species is often greater than that between different isoforms in the same species[22]. (3) As with maximum shortening velocity and ATPase activity, there is not so much difference between rabbit muscle and human muscle[23]. (4) Moreover, as rabbit skeletal muscle has been used in physiological experiment for a long time, it is very convenient to use it as experimental material.

In the present study, temperature was rather low compared with that of the human body. As in a cold environment, the temperature of the end of a digit could be about 20°C at most[24], our experimental conditions were necessary to clearly study the low temperature effect on muscle. Another reason is that glycerinated muscle fiber is susceptible to high temperature. i.e. experiments with it are impossible at 30°C or above.

The results of the present study suggest some causes of muscle damage at low pH and temperature: longer time to relaxation, shorter time to maximum stiffness, different decrease ratios of fast and slow muscle. Although the glycerinated muscle fibers used in this experiment were useful to change the environment of contraction proteins since the solutions easily penetrated to the binding between actin and myosin, these fibers would deteriorate with repetitive contraction. Studies are necessary with intact muscle fiber which would be stronger, more stable and closer to muscles *in vivo* than glycerinated muscle fiber.

Acknowledgement

The authors thank Dr. Haruo Sugi and Dr. Takakazu Kobayashi (Teikyo University) for their technical assistance, Dr. Joseph Hoh (University of Sydney) for correcting manuscript, and Dr. Shinichi Sawada, Dr. Susumu Saito, Dr. Tsutomu Okuno and Dr. Kazuo Kanada of the National Institute of Industrial Health for supporting the present experiment.

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Industrial Health 2002, **40**, 371–374 **Short Communication**

Involvement of Caspase 3 Mediated Apoptosis in Hematopoietic Cytotoxicity of Metabolites of Ethylene Glycol Monomethyl Ether

Akira TAKAGI[1], Takayuki YAMADA[1], Koji HAYASHI[2], Yuusuke NAKADE[1], Tetsuhito KOJIMA[1], Junki TAKAMATSU[3], Eiji SHIBATA[1], Gaku ICHIHARA[4], Yasuhiro TAKEUCHI[5] and Takashi MURATE[1]*

[1] Department of Medical Technology, Nagoya University School of Health Sciences, Daiko Minami 1-1-20, Higashi-ku, Nagoya 461-8673, Japan

[2] Central Examination Laboratory, Gifu Red Cross Hospital, Gifu, Japan

[3] Departments of Blood Service,

[4] Occupational and Environmental Health, Nagoya University Graduate School of Medicine, Nagoya, Japan

[5] National Institute of Radiological Sciences, Chiba, Japan

Received January 4, 2002 and accepted May 23, 2002

Abstract: The hematopoietic toxicity of ethylene glycol monomethyl ether (EGME) and its metabolites, methoxy acetaldehyde (MALD) and methoxyacetic acid (MAA), was analyzed using human bone marrow cells from a lymphoma patient without bone marrow involvement and a human leukemia cell line, HL 60. After 24-hour incubation, the concentrations of 50 percent inhibition (IC_{50}) of human hematopoietic progenitor cells with MALD or MAA were 3 mM and 3.9 mM, respectively, and EGME (10 mM or more) did not show any cytotoxicity. IC_{50} (after 48-hour exposure) of MALD and MAA on HL 60 cells were 2.45 mM and 5.6 mM, respectively, suggesting that both hematopoietic progenitor cells and HL60 have a similar sensitivity. DNA ladder formation, a characteristics of apoptosis, was observed in MALD- or MAA-treated HL60 cells, but not in EGME-treated samples. Caspase-3 enzyme activity, the effector of the apoptotic process, was greatly enhanced with MALD treatment. The inhibitor of caspase-3 repressed cell death induced with MALD as well as MAA.

Key words: Ethylene glycol monomethyl ether, Apoptosis, Hematopoiesis, Caspase-3

Ethylene glycol monomethyl ether (EGME) is widely employed as an industrial solvent as well as diverse household products. The widespread production and use of EGME has resulted in many reports of human poisoning by accidental ingestion or occupational exposure.

Exposure of laboratory rodents to EGME has been found to produce a variety of toxicologic effects[1]. The toxicity reported for short-chain ethylene glycol ethers such as EGME or 2-ethoxyethanol (2EE) is dependent on the sequential metabolism of these compounds by alcohol and aldehyde dehydrogenase enzymes (mainly in the liver) with formation of the corresponding metabolites. In the case of EGME, methoxy acetoaldehyde (MALD) and methoxy acetic acid (MAA) are formed, and are eliminated predominantly in the urine[2].

In hematopoiesis, there have been a limited number of case reports[3] showing mild macrocytic anemia and leukopenia, and laboratory animals exposed with these metabolites showed bone marrow hypocellularity resulting in pancytopenia[4]. However, the data on human bone marrow cells remain insufficient, and the mechanism of their toxicity has not been studied extensively[5].

Here, we report the results of short-term culture of EGME and its metabolites on human hematopoietic progenitor cells

*To whom correspondence should be addressed.

as well as human leukemia cell lines. To determine the probable cause of the hematopoietic toxicity of these reagents, caspase 3 enzyme activity, which triggers apoptosis, was measured, and the effect of caspase 3 inhibitor was analyzed.

EGME and MAA were purchased from Sigma (St. Louis, Mo, USA). MALD was synthesized by Dr. H. Sajiki (Gifu Pharmaceutical University, Gifu, Japan) according to a previously described method[6]. The final solution contained 18% MALD in 50% methanol. The cell permeable caspase 3 inhibitor, Z-DEVD-FMK was from Calbiochem (San Diego, CA, USA).

Human bone marrow cells were obtained from a patient with non Hodgkin's lymphoma after obtaining the informed consent. Low density mononuclear cells (1.5×10^5/ml) were treated with various concentrations of EGME, MALD and MAA for 24 hours in RPMI 1640 medium with 10% fetal calf serum (FCS). After 24 hour incubation, a progenitor cell assay was performed basically as described previously[7]. The respective numbers of myeloid colony (CFU-GM) and erythroid colony (BFU-E) were counted under the inverted microscope.

A human leukemia cell line, HL60 was cultured in 10% FCS in RPMI 1640 medium. Cells (2 or 5×10^5/ml) were treated with various doses of EGME, MALD and MAA. Viable cell number was measured sequentially. In some experiments, caspase 3 inhibitor was added 30 minutes before the addition of MALD or MAA. DNA ladder formation was analyzed as described before[8].

Caspase 3 enzyme activity was measured using caspase 3 8 and 9 colorimetric protein assay kit (Medical Biological Laboratory Inc, Nagoya, Japan) under the instruction of manufacturer's guide. Briefly, 150 μg of protein lysate in 50 μl of lysis buffer was mixed with 50 μl of 2X reaction buffer. Then, the respective substrate (DEVD-pNA substrate for caspase 3, IETD-pNA substrate for caspase 8, and LEHD-pNA substrate for caspase 9, respectively) was added. After the incubation for 1–2 hours at 37°C, OD 405 nm was measured with a micro-titer plate reader. Fold increase of each caspase activity was determined by comparing the result with the level of the non-treated control.

Figure 1 presents the result of progenitor cell colony formation of human bone marrow cells after one day exposure of each reagent in suspension culture. In this typical experiment, about 400 colonies (including both BFU-E and CFU-GM) were formed out of 1.5×10^5 bone marrow cells without drug treatment. EGME (2, 5 and 10 mM) were not inhibitory for human progenitor cells. However, MALD and, to a lesser extent, MAA, inhibited colony formation in a dose dependent manner. The concentration needed for 50%

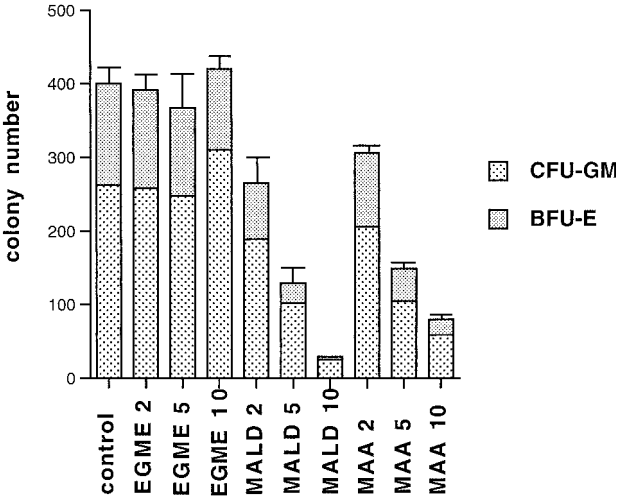

Fig. 1. Effects of EGME, MALD and MAA on normal human hematopoietic progenitor cells.

Inhibitory effects of EGME, MALD and MAA were shown compared to untreated control. After 24-hour incubation with various concentrations of each reagent (mM), mononuclear bone marrow cells (1.5×10^5 cells/ml) were plated in semi-solid medium in triplicates. After an appropriate time, colony numbers were counted as described in the Methods. The ordinate indicates the colony number/plate. Erythroid (BFU-E) and myeloid (CFU-GM) colony number are summed as the total colony number. To the total colony number, SD was added to each column.

inhibition of the control (normal) hematopoietic progenitor cell number (IC_{50}) of MALD and MAA were 3 mM and 3.9 mM, respectively. Both erythroid progenitor and myeloid progenitor were inhibited similarly with MALD or MAA.

Because of the difficulty in obtaining sufficient human bone marrow samples repeatedly, HL60, a human leukemia cell line was analyzed for further experiments. Figure 2A shows the relationship between viable cell number and the concentration of each reagent. MALD and MAA but not EGME inhibited the growth and survival of HL60 cells, which is consistent with the data on normal human hematopoietic progenitor cells. IC_{50} of MALD and MAA (two days' continuous exposure) were 2.45 and 5.60 mM, respectively. EGME did not show inhibition.

During the culture peroid, morphological changes such as chromatin condensation and cell shrinkage, which are unique to apoptosis, were observed (data not shown). DNA ladder formation was analyzed using HL60 cells treated with MALD. Figure 2B shows a typical experiment indicating the presence of 180 base pair ladder.

We analyzed the effect of MALD (as the representative of EGME related metabolites) on the caspase 3 enzyme activities of cells (Fig. 3A). Caspase 3 activation was clearly observed in MALD-treated cells in a dose dependent fashion. However,

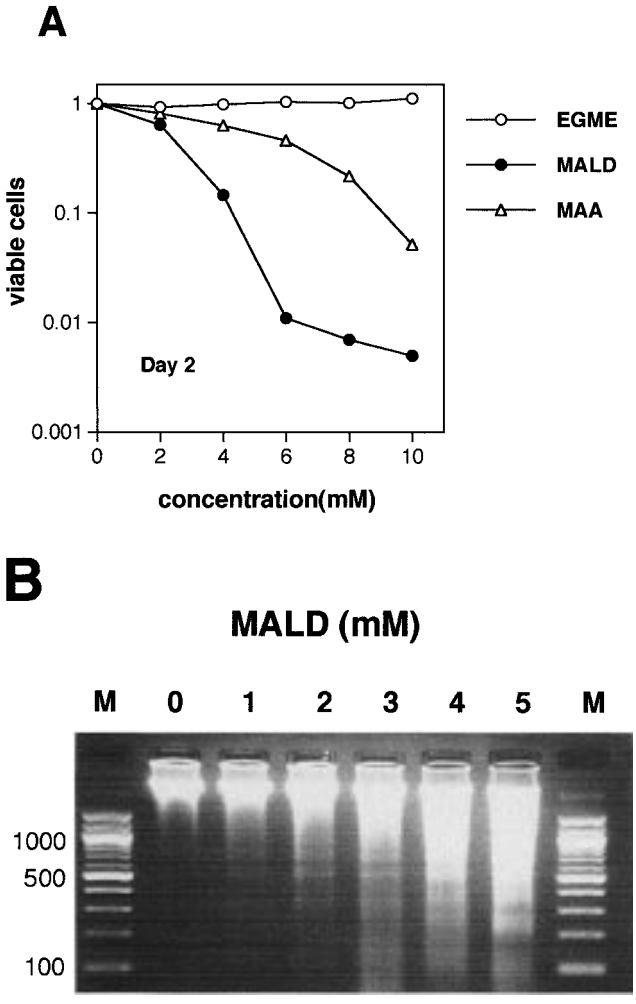

Fig. 2. A: Effects of EGME, MALD and MAA on human leukemia cell line, HL60.

HL 60 cells were treated with or without various concentrations of EGME, MALD or MAA. Fig. 2A illustrates semi-log presentation of the relative cell number on day 2 with each control group regarded as ratio 1.00.

B: DNA ladder formation of treated HL60 cells.

HL60 cells were cultured with various concentrations of MALD for 2 days. Total DNA was extracted as described in the Methods. DNA was electrophoresed in 1% agarose gel. M lanes are the molecular marker (100 bp ladder).

we did not observe activation of both caspase 8 and caspase 9, which located upstream of caspase 3. Finally, we attempted a rescue experiment using caspase 3 inhibitor, Z-DEVD-FMK. We can observe the significant preservation of cell viability with this inhibitor, especially with MALD treatment of 4 or 6 mM (Fig. 3B) as well as MAA treatment (data not shown).

In the present study, we showed the cytotoxicity of MALD and MAA on human hematopoietic progenitor but not that of EGME. It is of note that the mM order of metabolites is necessary for a short-term exposure experiment to observe significant hematopoietic toxicity. It can thus be argued that the concentration used in this study were too high to have direct relevance to the toxic effects these compounds might have in vivo. However, more sensitive assay such as Annexin V staining might have detected subtle and earlier changes in apoptosis. In our experiment, the dose-response curve was found to shift towards lower concentrations with incubation times. Thus, one may presume that a lower concentration should be active in chronic exposure.

Concerning the myelotoxicity of EGME, Teheux noticed the necessity of re-evaluation of an earlier in vitro study[9]. However, the elucidation of the mechanism of its toxicity is nevertheless important for the understanding of these compounds' toxicity. Our results suggest that the order of cytotoxicity is the aldehyde form as the strongest, followed by the acetic acid form, and then the ether form. However, the present results do not explain the apparent differences of in vivo cytotoxicity between various ethylene glycol ethers. Further analysis is necessary to explain these differences.

Till now, there are no data showing a dose-response relationship between EGME-related metabolites and human hematopoietic progenitor cells. Our data revealed that human hematopoietic progenitor cells have a sensitivity to EGME-related metabolites to that of the cell lines reported by Ruchaud *et al.*[5]. Our IC_{50} value for each metabolite in human leukemia cell line, HL 60, confirmed his report.

Recently, apoptosis has been regarded as an important physiological as well as pathological mechanism of the determination of a cell's fate. Ku *et al.*[10] reported the induction of apoptosis in a thymus and testis in vivo experiment. In the present study, the DNA ladder of around 180 base pairs, which is the characteristic of typical apoptosis, was observed in MALD treated HL60 cells.

The caspases are a family of enzymes that are involved in the process of apoptosis. These enzymes are composed of two hierarchical structures, in which an apical caspase (e.g., caspase 8 or caspase 9) triggers the partial digestion and activation of the effector caspases that are the main player of the destruction of various cellular proteins. Strong activation of caspase 3, the main downstream caspase, was observed with MALD treatment in a dose dependent manner. Because cell permeable caspase 3 inhibitor repressed MALD-induced apoptosis, caspase 3 would seem to be involved with MALD-induced hematotoxicity, although the upstream caspases, 8 and 9 did not show their activation in our system (data not shown). The remaining possibilities are that (1) other caspases, such as caspase 1 or caspase 11 is activated or that (2) other signaling pathways such as MAP kinase are mainly involved. Further analysis for the upstream trigger is necessary.

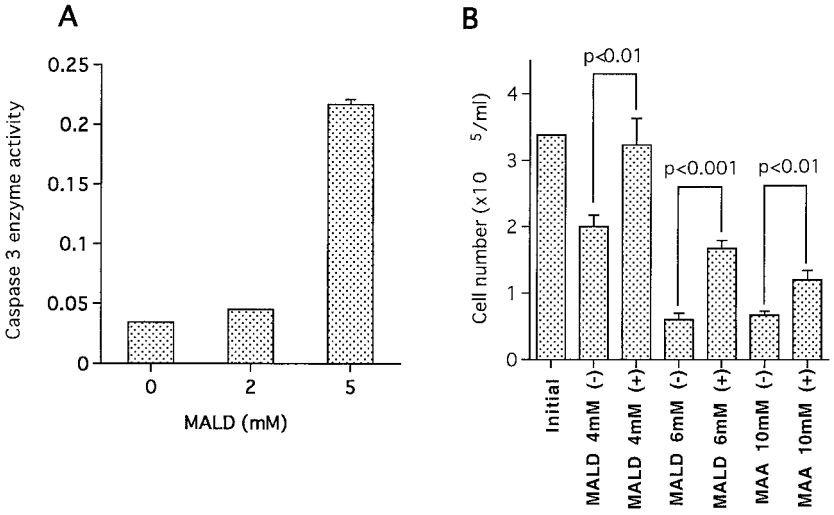

Fig. 3. A: Caspase enzyme activity of MALD-treated HL60 cells.
HL60 cells were cultured with 0, 2 or 5 mM of MALD for 36 hours. Caspase enzyme activity was measured as indicated in the Methods. The ordinate indicates the OD_{405} value representing the relative enzyme activity/150 μg protein. The mean ± SD from triplicates was described.
B: Rescue experiment with cell-permeable caspase 3 inhibitor.
Fifty μM of the caspase 3 inhibitor, Z-DEVD-FMK (illustrated as +), was added 30 min before addition of MALD or MAA of the respective concentration. On day 2, the viable cell number was counted. This concentration of Z-DEVD-FMK did not affect cell growth or cell viability (data not shown). The initial cell concentration (3.5×10^5/ml) was described at the far left column. The experiments were repeated several times with the similar results. The statistical significance (p value) was calculated with Student's *t* test.

Taken together, our results determined the IC 50 of MALD and MAA upon human hematopoietic progenitor cells as well as HL60 cells, and showed caspase 3 induced apoptosis was the probable cause of the hematopoietic toxicity of MALD and MAA.

The authors express their heartful thanks to Dr. H. Sajiki (Gifu Pharmaceutical University, Gifu, Japan) for providing us MALD.

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Industrial Health 2002, **40**, 375–381 **Short Communication**

Effects of *in Utero* and Lactational Exposure to Bisphenol A on Somatic Growth and Anogenital Distance in F_1 Rat Offspring

Kenichi KOBAYASHI*, Muneyuki MIYAGAWA, Rui-Sheng WANG, Soichiro SEKIGUCHI, Megumi SUDA and Takeshi HONMA

Department of Health Effects Research, National Institute of Industrial Health, 6-21-1, Nagao, Tama-Ku, Kawasaki 214-8585, Japan

Received May 30, 2002 and accepted August 27, 2002

Abstract: Bisphenol A (BPA), a xenoestrogen, has been reported to mimic the actions of estrogen or to affect the endocrine glands *in vivo* and *in vitro*. In this study, we examined whether *in utero* and lactational exposure to BPA altered the somatic growth and anogenital distance (AGD) of F_1 offspring (1, 3, and 9 weeks of age) *in vivo* in rats. Dams were orally administered with various doses of BPA (0, 4, or 40 mg/kg body weight (BW)/day) from gestation day (GD) 6 through postnatal day (PND) 20. There were no significant changes in body weight, liver weight, kidneys weight, testes weight, AGD, the ratio of AGD to BW, or the ratio of AGD to the cube root of BW in BPA exposed pups compared to the vehicle-exposed control. This suggests that prenatal and postnatal exposure (indirect exposure) to BPA (4–40 mg/kg/day, GD 6–PND 20) does not affect on somatic growth or AGD of F_1 generation of male and female rats.

Key words: Bisphenol A, Reproductive toxicity, Body weight, Anogenital distance, F_1 offspring, Rat

Bisphenol A (BPA) is very widely used in the manufacture of polycarbonate and epoxy resins, dental sealants, and other chemical products. BPA released from lacquer coating has been detected in food cans[1], and it has also been found in saliva collected from subjects treated with dental sealants[2]. Krishnan *et al.* have reported weak estrogenic action of BPA eluted from a polycarbonate bottle into medium during the autoclaving procedure. They showed that BPA increased the number of progesterone receptors and promoted the cell proliferation of a cultured cell line which originated from human breast cancer (MCF-7)[3]. BPA induced prolactin (PRL) release *in vitro*[4, 5]. It also increased uterine and pituitary weight, the serum PRL level and the number of immunoreactive PRL cells in ovariectomized Wistar rats[6].

Reproductive toxicity of BPA has been reported in mice and rats. Low-dose effects of BPA *in vivo* were observed in mice. BPA increased prostate and preputial gland weight, and decreased daily sperm production efficiency in male offspring prenatally exposed to BPA at 2 or 20 μg/kg/day from the gestation day (GD) 11 through GD 17[7, 8]. On the other hand, other investigators have failed to find such effects in mice offspring under identical experimental designs[9, 10]. Cagen *et al.* reported that normal reproductive development was observed in offspring born from mothers supplied with BPA in drinking water at a concentration range of 0.01 to 10 ppm (0.001–4.022 mg/kg/day) for 10 weeks, from the premating day (at 9 weeks old) to the weaning day, in Wistar rats[11]. In addition, it was reported that oral high- dose administration (320 mg/kg/day gavage) from GD 11 through postnatal day (PND) 20 resulted in no apparent change in male or female reproductive development in the F_1 offspring of Sprague-Dawley (SD) rats[12].

We determined the effects of BPA exposure from GD 6 through PND 20 on postnatal somatic growth and anogenital

*To whom correspondence should be addressed.

distance (AGD) in male and female SD rat offspring, because the effects of preweaning exposure on fetus growth and reproductive development have so far remained controversial. Examinations were performed on various clinical and reproductive parameters, including body weight (BW), main organ weight (liver, kidneys and testes), AGD, ratio of AGD to BW (AGD/BW), and ratio of AGD to cube root of BW ($AGD/BW^{1/3}$) at postnatal weeks 1, 3, and 9.

BPA (Bisphenol A standard, purity >99.8%, Cat#: 280-08561, Lot#: HCE9312) and corn oil (Cat#: 034-17015) were obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. A total of twenty-four pregnant female rats (Crj: CD (SD) IGS strain, 9 weeks of age) at GD 3 were purchased from Charles River Japan Inc. (Tsukuba), separately housed and maintained under controlled temperature (23 ± 1°C), humidity (55 ± 5%) and a 12-h light-dark cycle (0800–2000) throughout the study. The presence of a copulatory plug was considered to be GD 0. A standard laboratory diet (CE-2, CREA Japan, Inc., Tokyo, Japan) and drinking water were available *ad libitum*. Dams were divided into four equal-sized groups (6 pregnant rats/group) randomly, and weighed once a day from GD 3 through PND 20 (except for GD 4–5). The BPA-exposed groups were dosed by oral gavage with 4, 40 or 400 mg/kg BW/day of BPA in corn oil vehicle (10 ml/kg BW), once daily between 0830 and 0930, from GD 6 through PND 20, and the control group was given the same amount of corn oil during the same period. The highest dose, 400 mg/kg, was selected based on a study by Kwon *et al.*[12] reporting no detectable effects of BPA on maternal BW at 320 mg/kg/day from GD 11 through PND 20. The litter size was standardized to ten (male : female = 5 : 5, if possible) between 1000 and 1100 on PND 7 (1 week of age), and then all the culled offspring were used for examination as soon as possible after culling. On PND 21, offspring were weaned and thereafter males and females were housed separately per litter. The developmental parameters of offspring from various dose-treated dams were measured at 1, 3 or 9 weeks after birth. The body weight was recorded with an electric balance (Shimadzu, Kyoto, Japan). The anogenital distance (AGD) (mm) was measured with a digital caliper (Mitutoyo, Kanagawa, Japan) under euthanization by cooling on ice at 1 week or by ether inhalation at 3 or 9 weeks of age in F_1 offspring. AGD/BW (mm/g) and $AGD/BW^{1/3}$ ($mm/g^{1/3}$) were also calculated. A pair of male and female offspring from each dam were dissected at 3 or 9 weeks of age. The liver, kidneys and testes (male) were weighed at 9 weeks of age. Blood collected by decapitation at 1 week of age or sampled from the postcaval vein at 3 or 9 weeks was stored at –20°C for the determination of hormone levels (not reported here). The rest of the offspring were used for investigating behavioral effects (not reported here). The results were expressed as means ± SEM. The differences from the corresponding control group were statistically analyzed by analysis of variance, followed by Dunnett's test ($P<0.05$). The numbers of F_0 and F_1 rats used for examinations in each group are summarized in Table 1. In the control group, 1 female out of 6 was not pregnant. In the 40 mg/kg/day group, all the pups of a dam were found dead on PND 2. In the 400 mg/kg/day group, 4 dams out of 6 died on GD 21. All the pups born from 1 surviving dam in this group were found dead on PND 2. The 400 mg/kg/day BPA group was consequently excluded from further

Table 1. Treatment design and number of subjects examined

Group	Dose (mg/kg/day)	Dams[a]	Live birth[b]	No. of offspring tested			
				Age (wks)	1	3	9
Control	0	6[c]	12.8 ± 0.7	Male	6	5	5
				Female	8	5	5
BPA	4	6	13.5 ± 1.2	Male	9	6	4
				Female	8	6	5
BPA	40	6	11.5 ± 1.1	Male	1	5	4
				Female	10	5	5
BPA	400	6[d]	–	Male	–	–	–
				Female	–	–	–

[a]Six dams per group were dosed BPA or corn oil (10 ml/kg BW) by oral gavage from GD 6 through PND 20.

[b]The number of live offspring per litter on PND 1.

[c]One dam was not pregnant although the presence of a copulatory plug was verified.

[d]Four dams in the 400 mg/kg/day group died during the gestation period (–; Not examined).

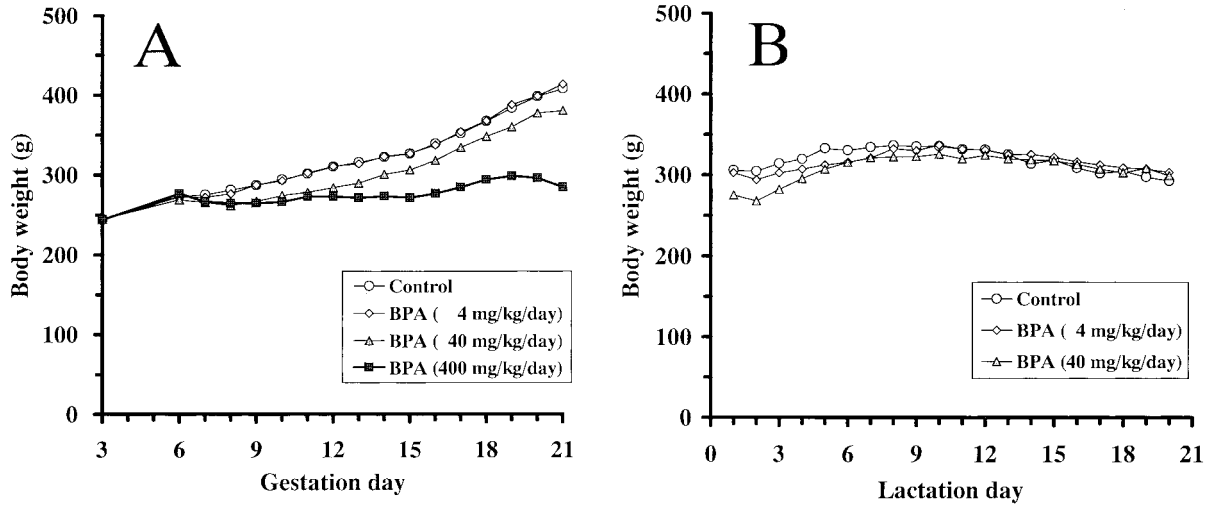

Fig. 1. Effects of exposure to BPA on maternal body weight during gestation (A) and lactation (B)
Each point represents the mean.

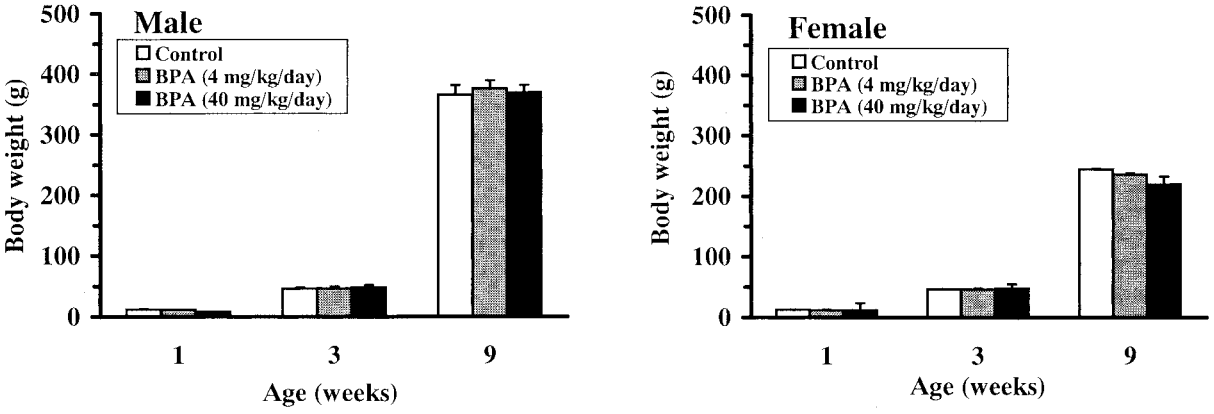

Fig. 2. Effects of maternal exposure to BPA on postnatal body weight of F_1 offspring at 1, 3 and 9 weeks of age
Each column and vertical bar represent the mean and SEM, respectively. There were no significant differences among groups.

analysis. The cause of death of these dams was not identified.

Maternal body weights during gestation are shown in Fig. 1A. The 400 mg/kg/day BPA group showed a marked reduction in body weight as compared with the control group, especially, in the third trimester of gestation. Maternal body weights during lactation are shown in Fig. 1B. The body weight changes in the various groups exhibited a similar pattern, although in the 40 mg/kg/day BPA group there was a transient reduction after delivery. There were no statistically significant differences among groups in the number of surviving offspring on PND 1 (Table 1).

In male offspring, there were no statistically significant differences in body weights among groups due to BPA exposure at 1, 3 or 9 weeks of age (Fig. 2). In female offspring, no statistically significant differences were observed in body weight, although the mean value for the 40 mg/kg/day dose group was slightly decreased at 9 weeks of age. There were no statistically significant effects on liver, kidney or testes (male) weights caused by BPA exposure at 4 or 40 mg/kg/day at 9 weeks of age (Table 2). Both in male and female offsprings, AGD were not significantly affected by BPA at 1, 3 or 9 weeks of age (Fig. 3). No treatment-related change was observed in AGD/BW or AGD/$BW^{1/3}$ ratios in male and female offspring at 1, 3 or 9 weeks of age (Figs. 4 and 5).

The fact that 4 dams out of 6 in the 400 mg/kg/day group died during the gestation period indicated that very high level exposure to BPA cause severe toxicity in pregnant rats.

Table 2. Liver, kidney and testes weights of offspring at 9 weeks of age

Organ	Sex	Dose of BPA (mg/kg/day)		
		0	4	40
Liver (g)	Male	17.52 ± 1.01	17.02 ± 0.70	17.10 ± 0.49
	Female	10.79 ± 0.71	10.41 ± 0.33	9.44 ± 0.64
Kidneys (g)	Male	3.22 ± 0.16	3.40 ± 0.20	3.28 ± 0.17
	Female	2.02 ± 0.16	2.12 ± 0.13	1.96 ± 0.13
Testes (g)	Male	2.78 ± 0.08	2.82 ± 0.18	2.90 ± 0.12

Values are the mean ± SEM. There were no significant differences for any groups.

Such maternal toxicity (reduction in maternal weight gain during gestation) was previously reported in rats exposed to BPA by gastric intubation at 160, 320 and 640 mg/kg/day[13]. They observed no dose-related maternal mortality in 160, 320 or 640 mg/kg/day groups, although they found a high mortality rate in the 1280 mg/kg/day group. The difference in the toxic effect of BPA observed in the two studies could be attributable to the difference in the purity of BPA (>95% *vs.* >99.8%). The duration of administration (GD 6–15 *vs.* GD 6–PND 20) and the preparation of the BPA (suspension in corn oil *vs.* solution in corn oil) also could be the causes of the discrepancy.

Testosterone from fetal testis develops Wolffian ducts and external genitalia with age, and AGD in males then exhibits a marked increase compared to that in females. AGD has therefore been used as a common index of reproductive and developmental toxicity, sensitively reflecting the status of the genital system and function in rodents. Vandenbergh *et al.* used the AGD/BWx100 value as an adjusted AGD index for BW or body size[14]. Gallavan *et al.* proposed to use the ratio of AGD to the cube root of BW as a better measure to evaluate the AGD, because body weight increases in a three dimensional manner whereas AGD increases in a linear manner[15]. Although we evaluated AGD including both of the adjusted measures, no differences were detected among treatment groups in any of the AGD indices (Fig. 3, 4, 5).

It was also demonstrated that low-dose BPA increased AGD after birth in CD-1 mice[16] and ICR mice[17]. The discrepancy between their studies and ours could be due to differences in the route and doses of chemicals, and animal species.

Cagen *et al.*[10] reported a study to confirm the effects of BPA in CF-1 mice, trying to replicate the experiments by Nagel *et al.*[7] and vom Saal *et al.*[8]. They carried out animal experiments under the almost same design with a larger number of groups, each consisting of a larger number of dams, with a wider range of doses (0.2–200 μg/kg/day) from GD 11 through GD 17. As a result, they found no effect of maternal exposure to BPA on F_1 mouse offspring, and concluded that BPA is not a selective toxic substance under their conditions. Evaluations of BPA effects at low doses have been performed in a multigeneration reproductive toxicity study. Tyl *et al.* examined the effects of exposure to BPA at dietary concentrations of 0, 0.015, 0.3, 4.5, 75, 750 and 7500 ppm (equivalent to 0, 0.001, 0.02, 0.3, 5, 50 and 500 mg/kg/day, respectively) on reproduction and development for three generations in rats. They concluded that there were no treatment-related low-dose effects and no evidence of a non-monotonic dose response on any adult or offspring parameters (including AGD) at concentrations of 0.015, 0.3, 4.5 and 75 ppm[18]. Ema *et al.* also reported negative results on the low-dose effects of BPA in a two-generation reproduction study. Rats were given BPA at 0.2, 2, 20 or 200 μg/kg/day by gastric intubation throughout the study beginning at the onset of a 10- and 2-week premating period, in F_0 males and females, respectively, and continuing through the mating, gestation, and lactation periods, for two generations. They observed no compound-related changes in clinical growth and signs, main organ weights including liver, kidney, testes or AGD/$BW^{1/3}$ under their conditions[19]. Furthermore, Kwon *et al.*[12] showed that high-dose exposure at 320 mg/kg/day from GD 11 through PND 20 resulted in no apparent change in male and female pubertal development and reproductive function in SD rats.

Our results were closely accordant with the findings of Kwon *et al.* The BW, liver weight, kidney weight, testes weight, AGD and AGD indices in BPA-exposed rats were not obviously changed by relatively larger amount of BPA exposure. The present study failed to find obvious effects on the somatic growth and reproductive development of the offspring due to maternal exposure to BPA at 4–40 mg/kg/day from GD 6 through PND 20, although the number of parameters used for evaluation in our experiment was smaller than those in other papers[9–12]. Some other indices are also

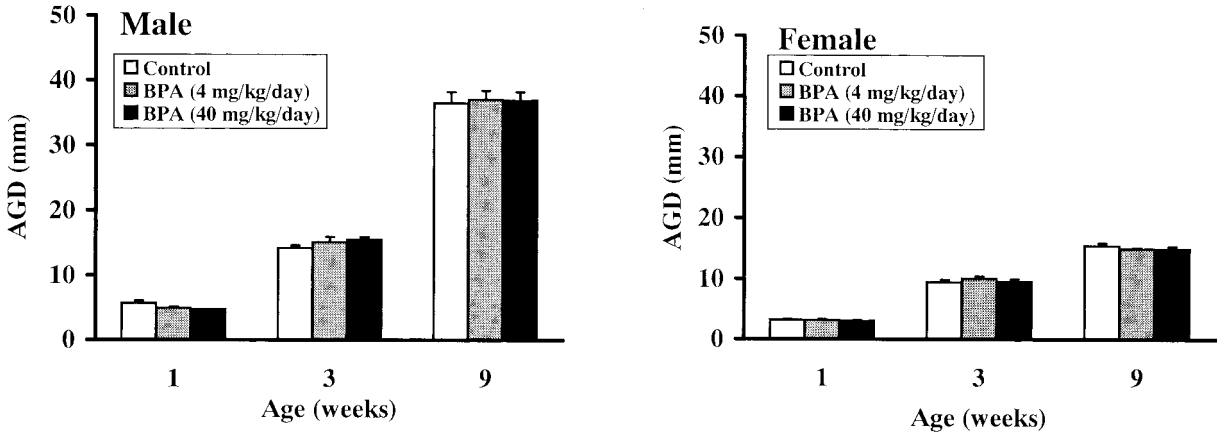

Fig. 3. Effects of maternal exposure to BPA on postnatal anogenital distance (AGD) of F_1 offspring at 1, 3 and 9 weeks of age
Each column and vertical bar represent the mean and SEM, respectively. There were no significant differences among groups.

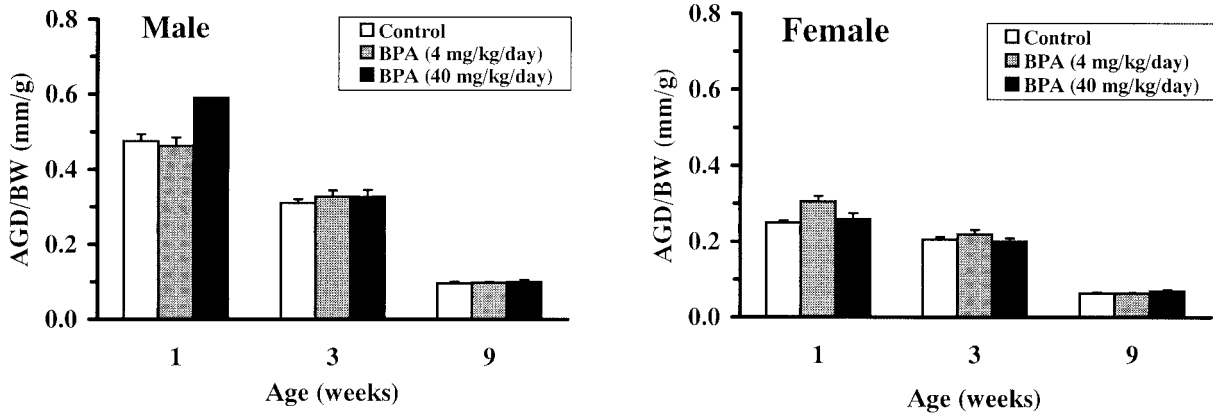

Fig. 4. Effects of maternal exposure to BPA on postnatal anogenital distance/body weight (AGD/BW) of F_1 offspring at 1, 3 and 9 weeks of age
Each column and vertical bar represent the mean and SEM, respectively. There were no significant differences among groups.

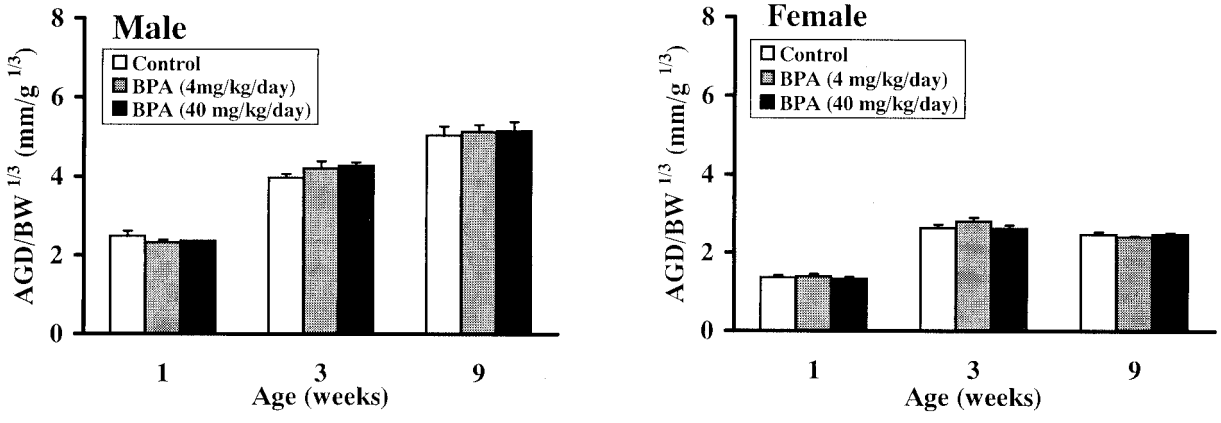

Fig. 5. Effects of maternal exposure to BPA on postnatal anogenital distance/cube root of body weight (AGD/$BW^{1/3}$) of F_1 offspring at 1, 3 and 9 weeks of age
Each column and vertical bar represent the mean and SEM, respectively. There were no significant differences among groups.

available to detect altered reproductive functions. In our previous papers, changes in the estrous cycle and ovulation have been shown to indicate disorders in the reproductive functions of female rats[20–22]. More extensive or detailed examination may result in finding some effects of perinatal exposure to BPA.

In conclusion, the results obtained in the present experiment suggest that prenatal and postnatal exposure to BPA does not affect the somatic growth and AGD of F_1 generation either in males or females. The effects of BPA exposure are, however, still incompletely understood and further work should be done to confirm the reproductive and/or developmental toxicity of BPA in rats.

Acknowledgments

We thank Mr. T. Murase for his helpful assistance and valuable advice. This study was conducted as a part of the contract research with the Ministry of Health, Labour and Welfare, which was supported by funds from the Ministry of the Environment.

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Industrial Health 2002, **40**, 383–387 **Case Report**

Concurrent Malignant Mesothelioma of the Pleura and Hepatocellular Carcinoma in the Same Patient: A Report of Five Cases

Claudio BIANCHI*, Lucia RAMANI and Tommaso BIANCHI

Laboratory of Pathological Anatomy, Hospital of Monfalcone, 34074 Monfalcone, Italy

Received October 29, 2001 and accepted June 14, 2002

Abstract: Five cases are reported in which malignant mesothelioma of the pleura and hepatocellular carcinoma co-existed in the same patient. The group included four men and one woman, aged between 58 and 86 years. The diagnosis was established at necropsy. In one case the association was clinically suspected. All mesotheliomas were asbestos-related. Liver cirrhosis co-existed in four cases, two of them positive for HCV markers. A lot of elements suggest that the above association is not a fortuitous coincidence. In particular, asbestos could favour liver cancerogenesis by inducing immune impairment.

Key words: Mesothelioma, Pleura, Liver carcinoma, Multiple malignancies, Asbestos, Immune impairment

Introduction

The association between malignant mesothelioma and other primary malignancies has repeatedly been described[1–26]. Multiple tumors may be a source of serious difficulties in the diagnosis. In addition, the association of more types of cancer is of interest from the etiologic point of view. When the two tumors are metachronous, the hypothesis has to be considered that the treatment adopted for the former malignancy, has induced/or favoured the development of the latter[13, 21–23]. But the most relevant point is that the development of different cancers in the same subject could indicate that such tumors share some etiopathogenetic factors. We report five cases of pleural mesothelioma associated with hepatocellular carcinoma, and we discuss the possible relationships between these two types of cancer.

Cases

The principal characteristics of the cases are summarized in Table 1.

Case 1

A 86-year-old man had been treated for recurrent right pleural effusions during the last eight months. The patient died for heart failure in February 1994. The necropsy disclosed a malignant mesothelioma of the right pleura (histological type mixed). Right lung, chest wall, pericardium, and peritoneum were involved by the neoplasia. Metastases were found in mediastinic and peripancreatic lymph nodes. Left pleura showed large pleural plaques. The liver showed macro- micronodular cirrhosis and a 2 cm large hepatocellular carcinoma. The patient had worked for 30 years (1937–67) in the shipyards of Monfalcone. Isolation of lung asbestos bodies after chemical digestion following Smith-Naylor method[27] showed 88,000 bodies/g dried tissue. Data about hepatitis markers were not available.

Case 2

A 72-year-old man was admitted in January 1996 for a right pleural effusion. Cytological examination of pleural fluid showed the presence of neoplastic cells. Liver ECT showed hepatomegaly and a 2 cm node. Other relevant findings were AFP 1,200 ng/ml, and negative HBV and HCV markers. A diagnosis of liver cell carcinoma was made,

*To whom correspondence should be addressed.

Table 1. Concurrent malignant mesothelioma of the pleura and hepatocellular carcinoma. Main features in five cases

Case N°	Sex	Age	Asbestos exposure data				Liver cirrhosis	HCV
			Occupational history	A. b.	P. p.	L. p.		
1	M	86	Shipyard worker	88,000	+	56	+	?
2	M	73	Shipyard worker	v.	+	46?	+	–
3	M	71	Sailor, various industries	n. v.	+	?	+	+
4	M	77	Shipyard worker	v.	+	59	+	+
5	F	58	Domestic exposure	70	–	46	–	?

A. b. = asbestos bodies: body amounts/gram of lung dried tissue; v. = visible in routine lung sections; n. v. = not visible in routine lung sections; P. p. = pleural plaques; L. p. = latency period.

and the patient was treated with Tamoxifene and intrapleural Bleomicin. The general conditions remained relatively good for one year. In September 1997, AFP level showed a dramatic increase (88,400 ng/ml). In the following months progressive deterioration occurred, and the patient died with gastroenterorrhagy in November 1997. A diagnosis of probably primary liver neoplasm with pleural metastases was made. At necropsy major findings were malignant mesothelioma of the right pleura (histologically epithelial type), large hepatocellular carcinoma in micronodular cirrhosis, small carcinoma of the urinary bladder, pulmonary asbestosis. The patient had worked as a shipwright at the Monfalcone shipyards for about 25 years after 1950.

Case 3

A 71-year-old man was admitted for left pleural effusion in March 1997. A biopsy of the pleura showed an undifferentiated neoplasia. Chronic liver disease co-existed with positivity for HCV marker. The patient died in June 1997 with diagnosis of pleural mesothelioma and HCV-related liver cirrhosis. The necropsy confirmed the clinical diagnosis; in addition a hepatocellular carcinoma, and large pleural plaques were observed. Mesothelioma, histologically mixed type, had metastasized at several sites (lymphnodes, liver, peritoneum, large bowel, adrenal, kidney). The lungs were involved by severe fibrosis, partly peribronchial and peribronchiolar in pattern, partly with large fibroelastotic scars substituting the parenchyma. Severe anthracosis co-existed. Asbestos bodies were not seen on routine lung sections. The patient had served as an engineer in the Navy for some years. Moreover he had worked in various industries.

Case 4

A 77-year-old man has been treated for HCV-related chronic hepatitis since 11 years. In August 1998 a liver biopsy showed a hepatocellular carcinoma. The neoplasia was treated by radiofrequency and chemioembolization. In October 1998 a thoracic CT showed a mass involving the soft tissue of left hemithorax associated with left pleural effusion. In the following months several left thoracenteses were performed, and intrapleural bleomycin was given. The patient died in September 1999 with the diagnosis of "HCV-related hepatocellular carcinoma, suspected mesothelioma of the left pleura". At necropsy, the co-existence of the two tumors was confirmed. In addition, micronodular liver cirrhosis, small pleural plaques of the right pleura, and pulmonary asbestosis were observed. Histologically mesothelioma was classified as mixed. The patient had worked as a welder at the Monfalcone shipyards in the period 1939–79.

Case 5

A 58-year-old woman underwent left pleurectomy for malignant pleural mesothelioma (epithelial type) in August 2000. Some months later the patient showed evidence of recurrence, and signs of peritoneal involvement. She died in April 2001, seven months after surgery. At necropsy, mesothelioma involved pleura of both sides as well as peritoneum. The liver showed a small whitish nodule, some millimiters in diameter, with microscopic features of hepatocellular carcinoma. Asbestos bodies were not found on routine lung sections. A small amount of bodies (70/g dried tissue) were detected after isolation.

The patient had a definite history of asbestos exposure, since during the childhood she had cleaned the work clothes of her family members, the father and the uncle, both employed in the shipyards. She had worked in various workplaces, including a small factory in which transistor radios were carried out. It could not be ascertained if asbestos exposure has or not occurred in such place.

Discussion

In the past one of the basic criteria followed in the diagnosis of mesothelioma was to exclude the existence of other primary malignancies. In such a way the possibility of identifying the association of mesothelioma with other types of cancer was eliminated *a priori*[28]. Nevertheless, it is undoubtful that mesothelioma, like all the other varieties of tumors, may co-exist with other malignancies, and this occurrence has been documented by several researchers. Recently, Suzuki reported 32 cases of double tumor, observed in a very large series of mesotheliomas[26].

The current cases illustrate adequately the difficulties of the diagnosis. The co-existence of the two tumors was established at necropsy. However, the double malignancy was clinically suspected in one only of the five cases.

The development of two different primary tumors in the same subject raises the question if some relationship exists between the two types of cancer.

From the etiological point of view it is clear that in the present group all pleural mesotheliomas were asbestos-related. All the patients had histories of not trivial exposure to asbestos, with four of them showing objective signs of such an exposure (pleural plaques and/or high numbers of lung asbestos bodies). Liver carcinoma was related to viral infection at least in two cases, and possibly in the others. Asbestos-related mesothelioma is generally a tumor with a very long latency period[29]. In the present cases latency periods ranged between 46 and 59 years. Regarding liver carcinomas, the incubation period in the present cases cannot be calculated with precision. However, it is known that in hepatitis C virus-related liver carcinoma, the time interval elapsing between infection and the development of cancer is longer than 20 years[30]. This means that in the current cases the incubation periods of both tumors elapsed during the same decades. The tumors developed in different organs, but in the same years and on the same background.

A role of virus infection in the genesis of liver carcinoma is well recognized[30]. On the other hand a possible role of viruses has also to be considered for malignant mesothelioma: SV-40 has frequently been detected in mesothelioma tissues[31], the development of mesothelioma has been described in some patients with AIDS[32–35], and mesothelioma has been reported in association with malignancies, certainly or possibly virus-related, such as Kaposi sarcoma[14, 25], and lymphoproliferative disease[3, 7, 8, 12, 24].

Various data suggest that immunosurveillance plays a role in the genesis of cancer. Recent findings show that cytotoxic activity of peripheral-blood lymphocytes is inversally correlated with cancer risk[36]. As far as hepatocellular carcinoma is concerned, less recent researches indicated that patients with liver cirrhosis and with low natural killer cell activity were at increasing risk of hepatocellular carcinoma[37]. On the other hand it has been shown that exposure to asbestos is associated with reduced effectiveness of natural killer cells[38]. This suggests that in four of the present cases immune impairment, induced by asbestos, could have had a role in facilitating the evolution from liver cirrhosis to hepatocellular carcinoma.

The possible role of asbestos in the etiology of liver carcinoma has been proposed[39, 40]. This hypothesis did not receive attention. Nevertheless, various clues may be found in the literature, suggesting such a role. The classic studies of Selikoff and coworkers on the cohort of insulators of US and Canada, showed high mortality for various types of malignancy, including liver cancer[41]. This finding, observed when the death certificates were considered, disappeared when only selected cases investigated more in depth, were included. However, it has been emphasized[42] that the more suitable term of reference in these studies was the death certificate. In addition, some large studies on linkage of occupation and cancer incidence showed significant associations between primary liver carcinoma and some occupations characterized by heavy asbestos exposure[43]. Moreover, other researchers have reported significant increase of liver cancer[44], or increase of liver and biliary passages cancer[45], among asbestos workers.

In conclusion, some data suggest that the association between malignant pleural mesothelioma and hepatocellular carcinoma does not represent a simple coincidence. Clearly, the available epidemiological data are not sufficient to admit an etiologic role of asbestos in liver cancerogenesis. However, given the high incidence of liver carcinoma in the world and the widespread use of asbestos many countries had, the idea that asbestos might favour liver cancer deserves further attention.

Acknowledgments

The present study was partly supported by a grant from the Italian League against Cancer.

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