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Review

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The increasing cesarean rate globally and what we can do about it

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Summary **Cesarean sections sometimes save the lives of mothers and babies; however, they are excessively used compared to medical necessity, which is influenced by various factors that are explored in this article. Since, in most cases the risks of cesarean sections are greater than the benefits, particularly in cesareans that are not medically indicated, it is astonishing that cesarean surgery is the most common surgical procedure, taking away resources from medically necessary care. While economic incentive is counted among the reasons for the increasing cesarean trend, the situation is not so simple since many factors interact to cause the trend. Since reversal of the vaginal birth after cesarean (VBAC) trend downward is correlated with revised policy statements by *e.g.* American College of Obstetricians and Gynecologists (ACOG), which have since been partially moderated, it became much more difficult for medical institutions to provide VBACs due to concerns about liability. Although whether to give birth by cesarean delivery is a matter for informed consent, yet childbearing women are influenced significantly by their health service providers' opinions. Even though the World Health Organization (WHO) recommends the most peripheral level of maternity care for normal pregnancy and childbirth that is safe using midwives, yet the percentage of midwife deliveries is low. Among other things, it has been suggested that more childbirth by midwife delivery and in out-of-hospital settings can reduce medically unnecessary cesareans and the undue risks associated with them, and free up medical resources for those in need.**

Keywords: Cesarean sections, medicalization, VBAC, midwife

1. Introduction

The increasing cesarean section rate is a global issue in developed countries (Figure 1; Table 1). According to Figure 1, Mexico had the highest cesarean rate among 22 selected developed countries in 2007 or 2008 (latest year reported) (43.9%), followed by Italy (39.8%) and South Korea (35.3%). The U.S. cesarean rate was 31.8%. The three lowest rates were 13.9% in the Netherlands, 16.1% in Iceland and 16.5% in Finland. The remaining countries were clustered in the band between 32.7% and 19.8%.

The research indicates that generally there are more disadvantages than advantages to cesarean sections although they are medically beneficial in appropriate situations. Literature review of what the developed countries learned about the benefits and risks and increase of cesareans during this time can inform us to plan the strategy going forward.

2. Methods

Five databases were used in a search strategy to identify the relevant literature: PubMed, EBSCO, Science Direct, the Cochrane Library and Google Scholar, from 1990 through 2011 limited to the last two decades and current to be able to follow the recent trends. I reviewed the relevant literature accessible by internet from Japan and selected over 30 articles, books and surveys as research sources for this article. After review of the literature accessed through the foregoing databases, the research was updated by internet search.

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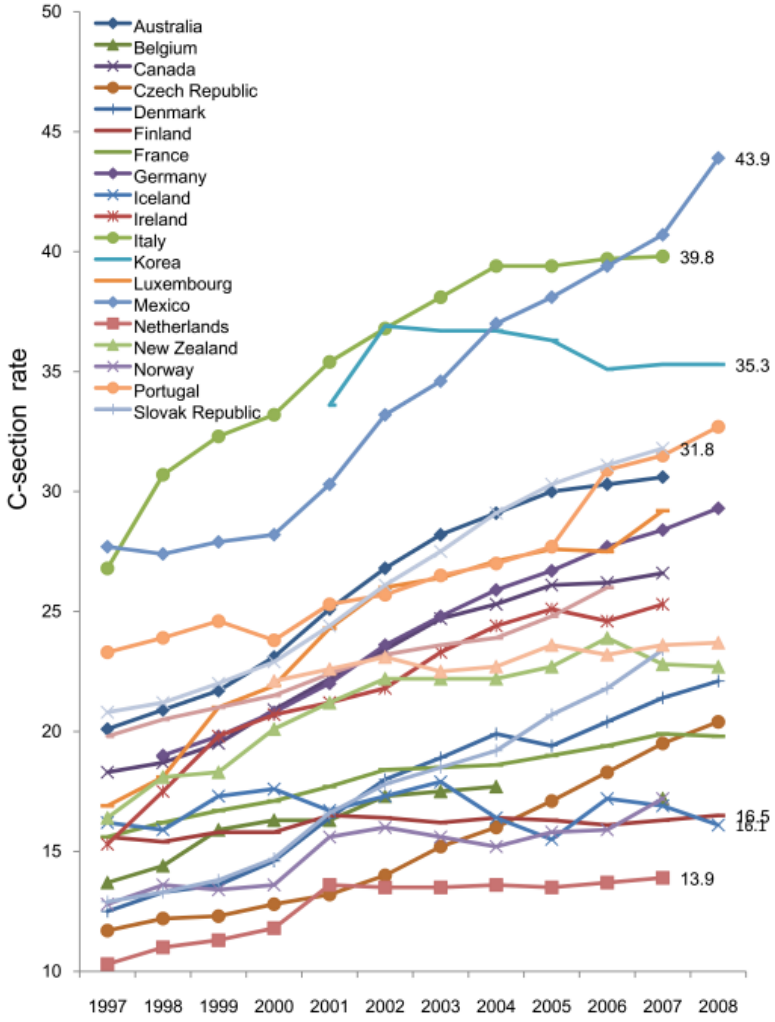

Figure 1. C-section rate within 22 developed countries. SOURCE: OECD HEALTH DATA, 2010 October (eliminated 9 countries from 31 OECD reported due to missing data for 3 recent consecutive years).

3. What are the trend, policy, and protocol?

3.1. *Steeply increasing trend*

Cesarean rates in the U.S. range from less than 10% for some caregivers and birth settings to over 50% for others (*1*,*2*) and cesarean section is the most common operating room procedure in the country (*3*). Various studies have shown that the rate of cesareans with no indicated medical risk is between 3% to 7% (*4*) and that between 4% to 18% of cesarean deliveries in 2006 were without medical indications therefor or on maternal request (*5*).

Between 1965 and 1986, the United States cesarean section rate increased from 4.5% to 24.1% (*1*) and the global rate rose from about 5% in developed countries in the early 1970s to more than 50% in some regions in the late 1990s (*6*). By 2004, the cesarean rate climbed to 29.1% in the U.S., an increase of more than 40% since 1996 reflecting an increase in the primary rate from 14.6% to 20.6% and a steep decline in the VBAC rate from 28.3% to 9.2%, with a repeat cesarean rate of almost 91% (*4*). By 2007 it rose to 32%, a nearly 60% increase (*7*).

On the other hand, the repeat cesarean rate rose by 28 percent from 1996 to 2005, when 92 percent of mothers with prior cesareans elected to undergo cesarean sections. Meanwhile, the global cesarean rate reached 25.7% as of 2010 (*3*). In 2008, it was estimated that one-third of deliveries in the U.S. were by cesarean, reflecting a steep rise in primary cesareans and a 72% decline in VBACs from 28% in 1996 to 8% in 2005 (*8*).

Many other countries also experienced a sharply rising cesarean section rate in recent decades. The medical indications for cesarean section are very subjective and culture bound such that there is a significant variation among countries with respect to cesarean rates for particular medical indications. Also, the country differences are salient regarding the rate at which particular common indications for cesarean birth apply to childbearing women (*9*).

Among the various countries compared by Sakala,

Table 1. C-section rate from 1997 to 2008 within 22 developed countries.

Country	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008
Australia	20.1	20.9	21.7	23.1	25.1	26.8	28.2	29.1	30.0	30.3	30.6	-
Belgium	13.7	14.4	15.9	16.3	16.3	17.3	17.5	17.7	-	-	17.2	-
Canada	18.3	18.7	19.5	20.9	22.2	23.4	24.7	25.3	26.1	26.2	26.6	-
Czech Republic	11.7	12.2	12.3	12.8	13.2	14.0	15.2	16.0	17.1	18.3	19.5	20.4
Denmark	12.5	13.3	13.6	14.6	16.4	18.0	18.9	19.9	19.4	20.4	21.4	22.1
Finland	15.6	15.4	15.8	15.8	16.5	16.4	16.2	16.4	16.3	16.1	16.3	16.5
France	15.6	16.2	16.7	17.1	17.7	18.4	18.5	18.6	19.0	19.4	19.9	19.8
Germany	-	19.0	19.8	20.8	22.0	23.6	24.8	25.9	26.7	27.7	28.4	29.3
Iceland	16.2	15.9	17.3	17.6	16.7	17.3	17.9	16.4	15.5	17.2	16.9	16.1
Ireland	15.3	17.5	19.8	20.7	21.2	21.8	23.3	24.4	25.1	24.6	25.3	-
Italy	26.8	30.7	32.3	33.2	35.4	36.8	38.1	39.4	39.4	39.7	39.8	-
Korea	-	-	-	-	33.6	36.9	36.7	36.7	36.3	35.1	35.3	35.3
Luxembourg	16.9	18.1	21.0	21.9	24.3	26.0	26.4	27.1	27.6	27.5	29.2	-
Mexico	27.7	27.4	27.9	28.2	30.3	33.2	34.6	37.0	38.1	39.4	40.7	43.9
Netherlands	10.3	11.0	11.3	11.8	13.6	13.5	13.5	13.6	13.5	13.7	13.9	-
New Zealand	16.4	18.1	18.3	20.1	21.2	22.2	22.2	22.2	22.7	23.9	22.8	22.7
Norway	12.8	13.6	13.4	13.6	15.6	16.0	15.6	15.2	15.8	15.9	17.2	-
Portugal	23.3	23.9	24.6	23.8	25.3	25.7	26.5	27.0	27.7	30.9	31.5	32.7
Slovak Republic	12.9	13.3	13.8	14.7	16.6	17.8	18.5	19.2	20.7	21.8	23.4	-
Spain	19.8	20.5	21.0	21.5	22.4	23.2	23.6	23.9	24.8	26.0	-	-
United Kingdom	-	-	-	22.1	22.6	23.1	22.5	22.7	23.6	23.2	23.6	23.7
United States	20.8	21.2	22.0	22.9	24.4	26.1	27.5	29.1	30.3	31.1	31.8	-

Source: OECD HEALTH DATA, 2010 October (eliminated 9 countries from 31 OECD reported due to missing data for 3 recent consecutive years)

Portugal, Sweden and Japan, and especially Japan stabilized the most through 1985. Notwithstanding Japan's bucking against the increasing cesarean trend at that time, the rate has since increaseed (Figure 2). The cesarean rates between 1970 and 1987 were compared by Sakala for 21 countries as of mostly around 1985. As of 1985, the lowest rates were in Czechoslovakia and the Netherlands at 6.5 and 6.6% with Japan showing at that time a bit more than 7%. The highest was in Puerto Rico at 29.3%, followed by Brazil at 26.1%, and then followed by the U.S. and Canada at around 23% and 19% respectively. Nine countries fell in the range of 10% to 15%. Of all the countries compared by Sakala, the Netherlands was the only one with a relatively high home birth rate of around 1/3 (*9*).

Figure 2 shows the comparative trends in Japan from 1984 through 2008 for cesarean sections in clinics and hospitals. Please note that Figure 2 commences from 1984 for Japan, to show the dramatically increasing rate for hospital cesareans in Japan compared to cesareans in clinics, which is a phenomenon regarding which salient data is available in Japan during this time period, even though Figure 1 showing developed countries merely commences in 1997. The in-hospital cesarean section slope in Figure 2 is more steeply increasing and the difference is especially salient between 2002 and 2008. Please note that between 2002 and 2005, the clinic cesarean rate increased about the same as the historical rate and then between 2005 and 2008 leveled off; the in-hospital rate however, shows its steepest incline between 2002 and 2008.

The rate in public hospitals in Brazil and South American countries had reached 80% in the early two thousands (*5*). A WHO global survey in 2005 disclosed a median rate of 33% based on a study of eight countries in Latin America, with 55% in private hospitals. Of the 33%, 49% thereof were elective, 46% were intrapartum, 5% were emergency cesareans without labor and 30% thereof had a prior cesarean delivery history. Among women whose labor was induced, a median of 28% had a cesarean delivery. The caesarean rate was positively associated with severe maternal mortality and morbidity, after adjustment for risk factors, and with increased fetal mortality, and antibiotics in postnatal treatment, but higher rates did not indicate better perinatal outcomes (*6*).

In Peru, the health reform enacted in 1997 increased the rate of caesarean sections in the private sector from 28% to 53%, apparently due to monetary incentives for overuse (*1*). Villar, Valladares and Wojdyla found that in Latin America, while the median rate of cesarean delivery was 33%, it was 51% in private hospitals. The caesarean rate in the private sector more than doubled in 15 years from less than 30% to more than 60% in the mid-two thousands, while in the public sector cesarean sections remained almost constant in MOH hospitals (for unemployed or informal employees) and increased at a slower rate in ESSALUD (social security) hospitals. It was concluded that one reason for such discrepancy might be that there was incentive to overutilize cesarean sections in private hospitals. Doctors in public hospitals work for fixed fees, while doctors in private hospitals are paid by a fee-for-service basis. Moreover, the increasing number of cesarean sections raised mortality and morbidity in mothers and babies as well as costs (*6,10*).

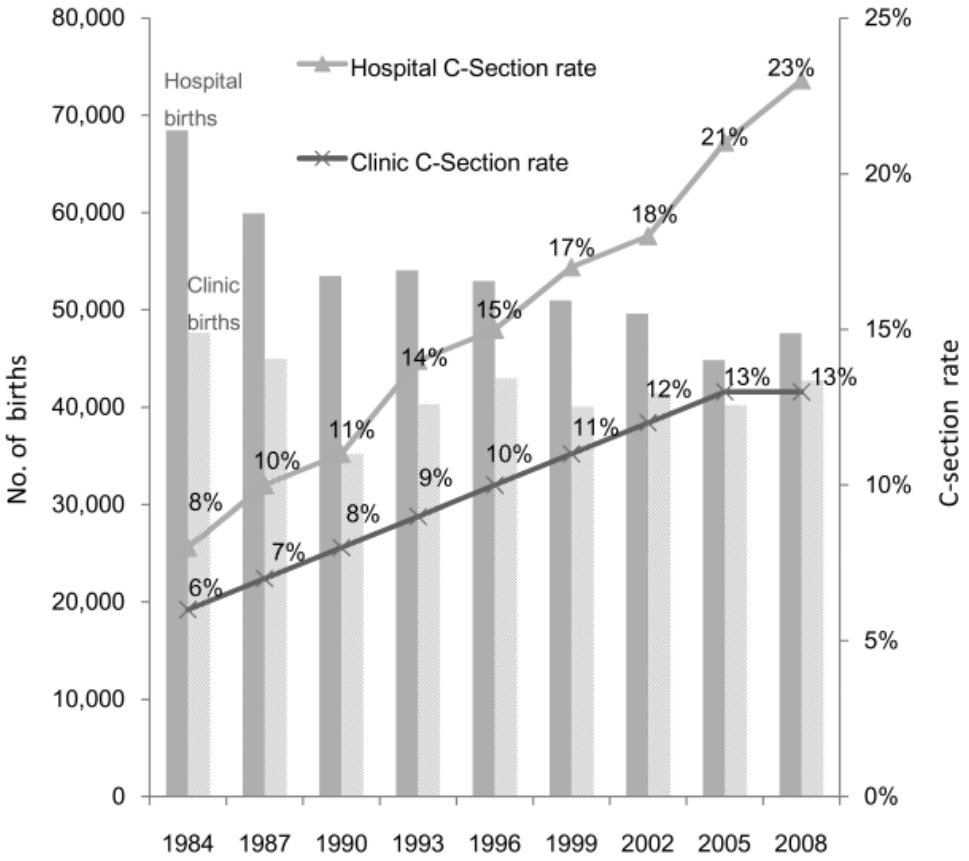

Figure 2. Births in Hospitals and Clinics. SOURCE: Report on Survey of Medical Institutions (September, 1986-2010, Ministry of Health, Labour & Welfare, Japan).

To give some context to the foregoing analysis, the increases of the cesarean rates in Peru were related to health reform which increased available funding but failed to provide good oversight or transparency. Prior to the reform, the probability of having a cesarean in a private hospital was only 7% greater than in a public hospital. According to WHO statistics, after the reform, 13% of Peruvian women had a cesarean section in 2000. More than 40% of deliveries were reported as non-institutionalized and mainly in homes, where the cesarean rate in the range of 1.4% to 1.9%. On the other hand, there was a cesarean rate of almost 50% for the 7% in private care facilities. While the cesarean rate increased in all facilities, the increase in the private sector was by 86% compared to 31% increase in the public sector (*10*).

Arrieta concluded that the fact that hospital ownership is the most significant non-medical factor in cesarean sections after the legislative reform suggests that physician incentives are playing an important role, although women's requests for cesarean sections are often psychologically driven and based on perceptions of safety and cultural and social factors as well (*10*).

In 2008, the cesarean rate was 20% in the UK, with many cesareans due to indications such as fear of pain or uncertainty of outcome (*11*).

In Australia, the rate of cesarean sections climbed from 22% in 1991 in private hospitals to 41% in 2006, with the rate in public hospitals rising from 16% to 28% and other interventions such as induction of labor, epidural anesthesia and pharmacological analgesia rising as well during the same period (*12*). It should be noted that medical interventions, such as labor induction, cause the likelihood of cesarean sections to increase (*13*), and there is a tendency for Electronic Fetal Monitoring (EFM) to do the same (*14*). During the same time period the cesarean rate was 14% in the Netherlands and ranged from 16% to 20% in Sweden and Finland (*12*).

A WHO study reported in a study of 24 countries that Chinese health facilities had the highest rate of 46.2% and China had an 11.6% rate of cesarean deliveries without medical indications (*15*).

3.2. *Shifting slope*

Due to questioning of the rationality of the trend to increasing cesarean sections, the rate in the U.S. stabilized, without significantly varying between 1986 and 1990 (*9*). There was a six year decline from 1990 through 1996, with cesarean delivery rates in the United States then rising from 21% in 1996 to 33% in 2007.

It would appear from a review of the comparison of the total cesarean delivery slope with the slope rate of the sharply rising (1989-1997) and then sharply falling (1997-2006) VBAC rate that the primary reason for the falling and then rising again cesarean rate was the steep incline and then decline in VBACs during the relevant period (*16*). In 1993, Stafford *et al.* hypothesized three reasons for the decline in cesarean deliveries between 1988 through 1992, for the first time after two decades of increase, namely: (a) literature critical of high cesarean section rates; (b) increasing public awareness of cesarean section practices; and/or (c) changing reimbursement policies of insurers (*17*).

Even so, while some of the foregoing factors may have had an effect on stabilization to some degree, considering the sharp reversal of the decline in cesarean deliveries, which correlated inversely with the changing VBAC practice and statistics, the changing VBAC rate would seem to be a controlling factor. This is particularly the case since a sharp increase in cesarean deliveries preceded the steep reversal and also inversely correlated with the changing VBAC practice and statistics (*16*). For example, from 1996 to 2004 the cesarean rate increased from 14.6% to 20.6% while the VBAC rate declined from 28.3% to 9.2%. During those years the cesarean rate increased by 40% to 29.1% (*4*).

A growing body of research literature has demonstrated the safety of VBACs, and policy statements from the leading professional association for obstetricians have supported VBAC under many circumstances (*9*). According to one study, best available evidence supports access to VBAC for most women with a previous cesarean delivery (*8*). Certain types of morbidity are higher with vaginal birth and there is some evidence that morbidity is higher for VBACs than repeat cesareans, with most studies finding maternal morbidity to be highest for unplanned cesareans, lower for planned cesareans and lowest for vaginal birth (*5*). Overall VBACs have the lowest morbidity, yet unsuccessful Trial of Labors (TOLs) ending in cesareans have the highest morbidity (*16*).

For most of the twentieth century it was believed that once a woman underwent a cesarean delivery, future pregnancies should be by cesarean delivery. The option for a woman with a prior cesarean to have a TOL was used more often in the 1980s through 1996, after which the number of VBACs declined. At the same time the cesarean rate rose from 21 % in 1996 to 33% in 2007 (*16*).

Until recently, since 1996, one-third of hospitals and approximately one-half of physicians no longer offered TOL. A survey of American College of Obstetricians and Gynecologists (ACOG) fellows showed that 26% stopped offering TOLs for Medicaid patients. VBAC rates are higher for women enrolled in HMOs who give birth at public rather than private hospitals (*16*). Still, as of 2004, nearly 91% of women with prior cesareans gave birth by cesarean (*4*). Repeat cesareans accounted for 35% of all cesareans in 1987 (*18*).

Seventy-nine percent of low-risk New Jersey women underwent repeat cesareans without TOLs between 2003 and 2005. At the same time that the TOL rate has been rapidly declining, the vaginal delivery rate after TOL has remained constant at approximately 74% (*16*).

Among low-risk women, the repeat cesarean rate increased to 89% by 2003. In one study 92% of women had repeat cesareans for their next delivery. TOLs have a success rate in the range of 60% to 80%. The risk of uterine rupture, which is the main reason given for reduced VBACs is less than 1% (*16*).

It has been suggested that concern over medical malpractice issues might also be a factor in the reduction of VBACs. In fact, ACOG members confirmed that 30% of obstetricians stopped offering TOLs or performing VBACs because of the fear of professional liability claims or litigation. In a survey of ACOG fellows, fear of litigation was among the primary reasons for performing cesarean deliveries (*16*).

According to a study by Stafford in California, repeat cesareans were at the rate of 91.9% for private insurance, compared to 75.2% for indigent services, with the rate of VBAC at 24.8% for indigent women compared to 8.1% of the privately insured. Comparative rates reflecting economic incentive based on payer source were associated no matter what the diagnosis leading to the cesarean section, whether breech presentation, dystocia or fetal distress, or other diagnosis (*19*). Overall, by 2010, the rate of repeat cesareans in the U.S. exceeded 90% (*20*).

4. What factors caused the increasing cesarean trend?

4.1. *Economic incentives and related factors*

Women of higher socio-economic class, better insured and/or cared for by private services are more likely to have cesarean delivery. Possible reasons are physicians' interest in economic gain (due to higher income or insurance coverage), defensive medicine (because private physicians bear personal risk of malpractice) and the need to deal with more scheduling pressure of doctors in private practice. In such connection, cesareans are correlated with doctor's scheduling in Brazil and scheduling in Canada and Italy. Also, it has been thought that house staff in public institutions may be held more strictly to conservative protocols and private physicians have a closer relationship with their patients and ironically value them more (as they are receiving less appropriate treatment) (*9*).

As a striking example, in Rio de Janeiro cesarean rates at four hospitals in 1977 and 1978 ranged from 14.9% at the facility serving the poor to 80.2% at the facility for private patients. In the late 1970s the

cesarean rates at nine Sao Paulo hospitals ranged from less than 25% for the indigent to about 75% for private patients. In 1980 and 1981, 7.5% of indigent and 49.6% of privately insured women out of a population of 6,000 childbearing women using a Brazilian hospital had cesarean deliveries (*9*).

Fifty-five percent of women in Brazil, from families earning more than $1,000 per month had a caesarean section and many lower to middle class women sought caesarean deliveries to avoid poor quality care and medical neglect from social prejudice. The factors prominently associated with whether or not a woman underwent a caesarean delivery were social power and affinity for medicalization among the subject women (*21*). The Hopkins study in 2000 showed that doctors tend to recommend cesarean delivery by taking advantage of women's concerns over potential complications arising from childbirth (*22*).

Although a prior study by Gruber, Kim and Mayzlin in 1999 related cesarean sections to the fee premium paid by Medicaid to physicians when a cesarean delivery is performed rather than a vaginal birth, concluding that such fee premium increased the probability of cesarean delivery within the range of 3.04% to 5.51%, when, such study was later replicated by Grant in 2008, it was concluded that the effect of financial incentives (fee premium of $1,000) is only around 1%, whereas on the other hand cesarean probabilities were higher with mothers having clinical risk factors and matching between privately insured mothers and physicians with predisposition to do caesareans. Grant said that his group's findings were consistent with reports of Blue Cross's unsuccessful efforts to lower cesarean section rates through financial incentives (*17*).

A WHO study found a strong correlation between cesarean sections and the economic incentive therefor of the relevant institution such that seven out of twelve private institutions showed evidence thereof, as compared to 5% of social security institutions and 24% of public hospitals (*6*).

A study by Stafford of cesarean sections performed in California in 1990 based on 1986 data revealed women covered by private insurance had a cesarean delivery rate of 29.1%, whereas indigent women had a rate of 15.6%, with the overall rate being 24.4% (*19*).

Another study by Leone, Padmadas and Matthews in 2008 based on analysis of six countries, Bangladesh, Colombia, Dominican Republic, Egypt Morocco and Vietnam, concluded that women of higher socio-economic background with better access to antenatal services are most likely to undergo a caesarean section, but that women who exchange reproductive health information with friends and family are less likely to do so (*22*).

Women's personal choices and institutional factors such as financial incentives and fear of litigation account for high rates of caesarean sections among the wealthy, according to studies by Behague, Victoria, & Barros in 2002, Gould *et al.* in 1989 and Rosmans *et al.* in 2006. In Egypt, the increasing rate of non-medically indicated caesareans has been driven by physician practice patterns and financial incentives in the private sector. In Bangladesh, the relatively high percentage within the public sector was attributed to emergencies with limited access to hospital birthing, compared with private caesarean driven by choice or supply side factors (*22*).

4.2. *Increasingly high technology in medicine and increasing medicalization of childbirth*

There is a relationship between the international trends for high-technology obstetrics and an increasing number of cesarean sections (*9,18*). In general, cesarean sections have been part of or a result of the significant general trend of intensified use of medical technology for childbirth in the U.S. Interestingly, skills and knowledge for turning babies have been retained in the industrialized countries of Holland, Sweden and Germany (*9*).

The Avon Longditudinal Study of Parents and Children study concluded that epidural use is associated with increased risk of emergency cesarean, while being in a preferred labor position decreased the risk therefore. In an American trial by Thorp *et al.* in 1993, the number of cesarean sections was increased when epidural analgesia was used. In addition, the relative risk of primiparous women having a cesarean section was found in a study by Tracy *et al.* in 2007 to be 11.4 times greater after epidural during labor, which study was aborted because of the ethical issues of having a control group of women receiving epidural where there is such a significant statistical difference. The Tracy study noted that contrary results exist in research by Eriksson, Olausson and Olofsson in 2006, which was distinguished on the basis that they used institutions with a 40% to 49% epidural usage rate as a referent group (*13*).

4.3. *Four major cesarean indicators are gray areas*

Sakala opined that "The vast majority of cesareans performed in the U.S. are … attributed to official 'diagnoses' that are ambiguous and/or for which a cesarean offers no or highly questionable benefit (*6*)". In particular, the four major indicators of uterine scar, obstructed labor, fetal distress and breech presentation are gray areas (*9*).

For example, the assumption that a uterine scar from a prior cesarean section has a high risk of rupturing during a subsequent labor and birth led to a standard U.S. policy of cesareans in subsequent

pregnancies, regardless of medical status and has been a large factor in the rising rates of cesarean birth (*9*). Thus, as noted above, in 1987, 35% of all cesareans were subsequent cesareans (*18*).

Second, cesareans are often performed in case of dystocia or obstructed labor (*6,16*). In 1987, dystocia accounted for 40% of primary cesareans (*18*). It should be noted that while in 1980, 1.1% of births in the U.S. were labeled as involving obstructed labor; by 1989 the figure had risen to 4.3%, whereas the percentage of abnormal labor climbed from 3.0% to 7.4% in the same period (*9*).

Third, likewise, the increasing rate of cesarean births is associated with diagnosis of fetal distress. Thus, while 1.7% of all births were designated as involving fetal distress in 1980, 10% were so designated in 1987 (*18*), and, by 1989, 8.8% were so designated (*9*). To a large degree, this rise generally is a function of growing reliance upon EFM (*9*). However, there are false positives of around 50% and an excess of cesareans resulting therefrom, also with lack of expected benefits. In addition, EFM is testing for events that occur only in 1% to 2% of births (*14*). Moreover, while EFM is monitoring of the fetal heart rate (FHR) to detect risk of perinatal mortality due to inadequate oxygen supply to the fetal brain, EFM has not reduced perinatal mortality or the risk of cerebral palsy. It should be noted that the false positive rate for cerebral palsy from EFM is a whopping 99% (*23*).

By way of example, auscultation and EFM were compared in a number of trials by Haverkamp *et al.* in 1976 and 1979, Kelso *et al.* in 1978, MacDonald *et al.* in 1985, Wood *et al.* in 1981, and Neldam *et al.* in 1986. While cesarean section rate was higher in all electronically monitored groups, there is little evidence that the increased interventions in the electronically monitored groups led to substantive benefits for the infants according to the study by MacDonald *et al.* in 1985 (*24*).

In fact, clinical trials including high risk patients showed that nurse attendants are of more benefit to maternal or fetal outcome, while, on the other hand, a 50% false positive rate doubles the cesarean section rate. While the measures are precise, the interpretation of change of FHR is not. Such false positives have a tendency to cause intervention in the birth process, which causes problems in some cases. At the same time, EFM has a tendency to enhance defensive medicine practice (*14*).

Fourth, the assumption that cesarean birth is safer than vaginal birth for all babies in the breech presentation led to nearly universal cesareans in such cases, while the skills of inverting a breech baby and facilitating vaginal birth of breech babies were dropped from the medical curriculum (*9*). In 1987, breech births were associated with 10% of cesarean deliveries (*18*).

4.4. *Creeping diagnostic standards over time*

Particularly in connection with gray areas, the diagnostic standards and criteria have changed over time due to a more high-tech medical environment and a more medicalized approach to childbirth and a tendency to manage birth within more tightly controlled norms, which increasingly drives the cesarean rate, because the percentages of the diagnoses to perform cesareans have significantly crept up over time. Over time, factors leading to a diagnosis to perform a cesarean were more plentiful, but perhaps the standards therefor have significantly changed due to sociological and iatrogenic rather than strictly medical reasons. Thus, the practice of obstetrics in a high technology and managed time environment with pharmacological aids may have caused physicians to diagnose basically similar medical facts with more of a predisposition to perform cesarean surgeries, or such evolving practice, itself, caused the symptoms for such diagnosis.

As an example, one midwife identified five instances of fetopelvic disproportion in over 1,000 births or less than one-half of one percent of all births, whereas 3% to 15% of all births are associated therewith in the medical literature. The gradual move away from midwifery, out-of-hospital settings and low technology obstetrics (changing labor positions; supporting companion encouraging opening up; laboring in a comfortable place with known trusted people) to high technology and time structured obstetrics practice might perhaps account for such statistical difference (*18*).

4.5. *Various miscellaneous factors*

The cesarean rate has been said to be driven by the interaction between mothers and their providers. For example, in a study in Brazil by Potter *et al.*, more than 80% of primiparous mothers anticipated a vaginal birth one month prior to the due date, but almost half of them and 66% in private hospitals ended up with a cesarean (*4*).

Other reasons given for the increasing cesarean rate include improved surgical techniques, providers' and patients' perception of the safety of the procedure, change in health systems, the supposed benefits of protection against urinary incontinence, prolapse and sexual dissatisfaction, patient demand (*6*), and physician practice patterns (*22*). Also, it was reported by Declercq *et al.* in 2006 and the National Collaborating Centre for Women's and Children's Health in 2008, that a substantial proportion of cesarean sections in 2005 were performed because of caregivers' judgment and concern about a large fetus. Yet, according to studies by Chauhan *et al.* in 2005, Coomarasamy *et al.* in 2005, Pattinson and Farrell in 1997 and Rouse and Owen in 1999, the conclusion to perform a cesarean section because of concern about

a large fetus is not supported by the best research (*8*). In addition, another reason for the increasing number of cesarean births is the anxiety of physicians and mothers due to the increased use of obstetrical screening technologies and interventions, including for example EFM and labor inductions (*9*).

4.6. *Sociology of medicine type reasons*

Reasons for the increasing cesarean rate include, among others, under-use of care that can enhance the natural progress of labor and childbirth, such as a labor support companion, encouraging upright or moving positions during labor, rather than on the back (which inhibits labor), ensuring expectant mothers are well-rested and well-nourished while giving birth; the willingness of some caregivers to move to cesarean section before trying measures that may avoid the surgery, for example, by failing to attempt to turn babies in a breech position in late pregnancy or by failing to allow more time for a vaginal birth to occur due to institutional pressures; pressures on caregivers to practice "defensive medicine"; failure to offer women with a previous cesarean section a choice of VBAC, loss of skills or unwillingness to offer vaginal birth to women in some situations, *e.g.* breech birth or twins, the growing perception that a cesarean section is "safe" (*2*); casual attitudes about cesarean sections (*8,23*), low priority of enhancing women's own ability to give birth, limited awareness of harms that are more likely with cesarean section, defensive medicine, and incentives to practice in a manner that is efficient for providers (*25*).

4.7. *Midwives perspective*

From the midwives' perspective, many women receive cesareans due to pseudo-problems, to easily preventable problems or those that might be solved through less drastic measures. Sakala opines that midwifery knowledge and practice are based more directly on the interests, needs and circumstances of childbearing women as compared to obstetrical knowledge and practice (*9,18*). Thus, independent midwives, particularly, can construct the meaning of birth and practice maternity care largely unconstrained by prevailing medical practices since they have the opportunity to develop a women-derived and centered body of knowledge and practice of childbearing reflecting women's subjective experience, as distinguished from externally imposed obstetrical models. Thus, midwifery results in individualized care in dignity with respect, giving women a primary role in informed decision making, emphasizing health promotion and illness prevention, minimizing technological intervention and iatrogenesis and addressing physical, psychological and social issues of childbearing women (*18*).

5. Cesarean section protocols and health policy guidelines

The International Federation of Gynecologists and Obstetricians (FIGO) stated that:

"FIGO considers surgical intervention without a medical rationale to fall outside ... best professional practice. Caesarean delivery should be undertaken only … to enhance the well-being of mothers and babies and improve outcomes (*22*)."

"At present, because hard evidence of net benefit does not exist, performing cesarean delivery for nonmedical reasons is not ethically justified (*4*)."

However, the guidelines from ACOG leave it more up to the belief of the physician involved, as follows: "In the absence of significant data on the risks and benefits of cesarean delivery … if the physician believes that cesarean delivery promotes the overall health and welfare of the woman and her fetus more than vaginal birth, he or she is ethically justified in performing a cesarean delivery (*4*)".

National Healthy People 2010 objectives call for a substantial decrease in the cesarean rate and an increase in the rate of VBACs from 2000 to 2010, from the U.S. Department of Health and Human Services 2000 (*8*).

In the past, WHO recommended that optimal national cesarean rates were in the range of 5% to 10%, and that rates above 15 percent are likely to do more harm than good (*8*) and that the maximum caesarean rate should not exceed 15% (*22*). Various programs and policies have been proposed or implemented to reduce cesarean rates (*1*). A WHO study concluded that caesarian sections should be performed when a clear benefit is anticipated that would compensate for additional cost and risk (*15*).

In the UK, after a three-day conference on maternal request cesareans, the National Institute of Health (NIH) did not recommend against medically unnecessary cesareans (*11*).

In 1999, the ACOG changed its practice guideline encouraging VBAC to a recommendation that women should be offered TOL if there are no contraindications, and that such TOL should be performed only in institutions equipped to respond to emergencies where physicians able to perform cesareans are immediately available. Concern over medical malpractice played a role in the adoption of the guideline (*16*), and this writer would suggest also in the reaction to the guideline.

NIH in its 2010 Consensus statement stated that VBAC is a reasonable option, that the decision whether to undergo one should be made jointly by the childbearing woman and her physician after informed consent regarding risk assessment and that the woman's preference should be honored as much as possible (*16*).

ACOG's guidelines, under which one third of hospitals and doctors had blocked VBACs since the time of the trend re-reversal (from upward to downward), were then eased following NIH's Consensus statement. The new guidelines declared that cesareans are a safe and appropriate option for most women, even including those giving birth to twins or with two prior cesareans, and that childbearing women with prior cesareans should be informed of the pros and cons and decide whether they want to try. It was reported that women with prior cesareans try labor and between 60% to 80% successfully give birth vaginally (*26*,*27*).

ACOG's guidelines, however, still continued to stress that women attempting vaginal birth after a prior cesarean section should labor in a facility that is equipped to handle emergency care. However, under the new guidelines, a trial of labor can be made even if such emergency resources are unavailable if the childbearing woman and her physician know and plan the logistics of the community medical resources in advance considering incremental risk. Also, it was declared that a woman cannot be forced to undergo a repeat cesarean (*26*,*27*). It was reported that ACOG's new guidelines also stated that if such emergency resources are unavailable, women should "be allowed to accept increased levels of risk" if they are made aware of the potential dangers (*28*).

More generally, WHO has said that midwives are generally most appropriate to ascertain the risks of normal pregnancy, as follows: "The midwife appears to be the most appropriate and cost effective type of health care provider to be assigned to the care of normal pregnancy and normal birth, including risk assessment and the recognition of complications…"; "However, in many developed and developing countries midwives are either absent or are present only in large hospitals where they may serve as assistants to the obstetricians (*24*)".

6. Do the risks of medically unnecessary cesarean deliveries outweigh the benefits?

A substantial proportion of cesarean section deliveries involve medical risk for mothers and infants without medical benefit (*9*). Variations in cesarean rates do not closely correspond with variation in the risk status of the populations being served, but rather are associated with a large number of nonmedical variables. Nonmedical factors include maternal, medical system and physician factors (*9*,*18*). Maternal mortality is two to seven times higher, and morbidity five to ten times higher, in cesarean sections compared to vaginal delivery. Women undergoing cesarean sections have more pain than women delivering vaginally, longer and more difficult postpartum recovery, a higher likelihood of complications and cesarean sections in subsequent pregnancies, and more difficulty in conceiving after cesarean sections, as well as greater likelihood of stillbirth and miscarriage in subsequent pregnancies. One study by MacDorman, Declercq, Menacker, & Malloy in 2006 concluded that neonatal mortality for cesarean deliveries was 2.9 times greater than for vaginal deliveries in women with no medical risk factors. There is more prevalent respiratory distress syndrome and persistent pulmonary hypertension in surviving neonates after cesarean delivery compared to vaginal delivery, followed by more childhood asthma, but less infant injuries (*5*).

Even though it is thought that that 85% to 90% of pregnancies and births can safely take place by vaginal delivery, one quarter of childbearing women are told otherwise. As of 1986, the 24.1% cesarean rate in the U.S. substantially exceeded the estimated rate with medical benefits of 6% to 16.5% (*9*), the 5% to 10% optimal rate set in earlier editions and the maximum 10 to 15% rate previously recommended by WHO in 1985 (which has since more modestly suggested regions might want to set the rate between 5% to 15% or set their own standards) (*22*,*29*), as well as the optimal rate for industrial nations of about 7% according to a study by Francome and Savage (*9*).

A WHO study conducted through 2008 concluded that absent medical indication therefor, cesarean delivery has an increased risk of 280% for severe adverse short-term outcomes for the mother as compared to spontaneous vaginal delivery (42/1,000 compared to 15/1,000, respectively) and nearly six times as much (adjusted odds ratio 5.93, 95% confidence interval 3.88-9.05) if before labor onset, but after labor onset fourteen times as much (adjusted odds ratio 14.29, 95% confidence interval 10.91-18.72) (*15*).

According to Childbirth Connection, cesarean section is riskier than vaginal delivery in 33 areas and vaginal birth is riskier than cesarean delivery in four areas (*1*,*3*). Among others, the risks in cesarean sections include physical problems to mothers, including but not limited to maternal death, emergency hysterectomy, hemorrhage, blood-clots and stroke, bowel obstruction, injuries from surgery, infection (*1*,*3*,*8*,*15*), antibiotic resistance (*15*), pain, including ongoing pelvic pain; emotional problems to mothers, including, poor birth experience, later contact between mother and baby, unfavorable early reaction of mother to baby, depression, psychological trauma, poor overall mental health and self-esteem and poor overall functioning; reproductive problems for mothers, including but not limited to ectopic pregnancy, infertility, reduced fertility, placenta previa, placenta accrete, placental abruption and rupture of the uterus; concerns about babies in future pregnancies, such as premature, low-weight or physical abnormality (malformation) or central nervous system injury (to brain or spinal cord), stillbirth or death of infant; and risks to health of babies, including but not limited to, getting cut during surgery, breathing problems, childhood and adult asthma and reduced

breast-feeding. Risks in vaginal birth are perineal pain, incontinence, and nerve injury in babies (*1,3,8*).

There is evidence from studies by Allen, O'Connell, Liston & Baskett in 2003, Ecker in 2004 and Murphy, Liebling, Verity, Swingler, & Patel in 2001 that medically unnecessary caesarean sections could increase morbidity risks to mother and newborn (*22*). Cesarean sections also cost a lot more (*30*).

Dissatisfaction with childbirth is well-documented for cesarean delivery, which can cause postpartum depression; negatively affect perception of the newborn, with less positive reactions impeding infant cognitive and socio-emotional development, physical growth and health, parenting behavior and likelihood to choose to have another child. Women delivering by cesarean delivery provide less tactile stimulation, caretaking and intimate play with their babies within the first five months. Ironically, dissatisfaction may also lead to a lawsuit, while litigation defensiveness has been explained as one of the reasons why doctors perform cesarean sections (*5*).

Passage of the newborn through the birth canal helps expulsion of fluids from the baby's lungs facilitating early breathing efforts (*11*) and immunological defense (*8*). In addition, there may be an association between cesarean section and vulnerable child syndrome (*9*). Babies born by cesarean section are reported to have a greater risk for asthma and allergy, diabetes mellitus, childhood leukemia and testicular cancer (*31*).

Needless to say, medically unnecessary cesarean surgeries are a huge waste of medical resources (*9*). WHO reported in 2010 that the global cost of excess cesarean sections was estimated at approximately US$2.32 billion. Money spent on medically unnecessary cesarean sections must necessarily be taken away from money to fund necessary or desirable medical care for other medical conditions or for medically necessary cesareans that is unavailable for such reason (*30*). Some services in the U.S. have been able to considerably reduce cesarean rates without adversely affecting perinatal outcomes. Other nations with similar populations have been able to achieve similar or better perinatal outcome indicators with much lower cesarean rates. Furthermore, in the U.S. and abroad, services skilled in and committed to low-technology approach have maintained excellent outcomes and cesarean section rates below 2% (*9,18*). In fact, in Vienna, the clinic Ignaz Semmelweiss Frauenklink had a cesarean section rate for the 20-year period from 1966 through 1985 of 1.3%, compared to 8% in the rest of Vienna, even declining from first to second decade against the trend in the rest of the developed world (*18*).

7. Solutions and alternatives

Among solutions to a perceived excess of medically appropriate cesarean surgeries, the following basic strategies have been proposed and pursued: (a) resistance by child-bearing women and their advocates; (b) managed care strategies; and (c) more midwife birthing and out of hospital settings (*9*).

With respect to (b), above, one example is a hospital program requiring a second opinion, objective criteria for the most common indications, review of all cesarean sections and reporting of individual physician's rates (*9*).

As regards (c), above, American women beginning labor with midwives and/or in out-of-hospital settings have attained cesarean section rates that are considerably lower than similar women using physicians in hospitals. Moreover, groups of women at elevated risk for adverse perinatal outcomes have attained excellent outcomes and cesarean rates well below the general population rate with these care arrangements. One assessment by Rooks *et al.* found that the cesarean section rate in out-of-hospital centers was 4.4% at a time when the national rate was more than 20% (*18*). This cesarean reduction involved no compromise in mortality and morbidity outcome measures. Similarly, supportive labor companions or childbirth assistants are associated with a favorable effect on cesarean rates in several countries. In one trial with a doula present by Kennell *et al.* there was an 8% cesarean rate as compared with 13% with a silent observer and 18% with neither (*9,18*).

In connection with (c), above, Sakala concluded: "Because of the dim prospects for rational reduction of cesarean section rates with the prevailing medical care system, a growing number of analysts and organizations … recommend a third approach to the problem: midwives should have a much greater role in the care of childbearing women, and midwifery should be an autonomous profession…"; "Therefore, the most effective solution to the pandemic of medically unnecessary cesarean births is to demedicalize birth, and to limit the involvement of obstetrical specialists and acute medical settings to the case of genuine medical need…"; "Supporting and strengthening midwifery care and designating midwifery care as the most appropriate form of care for health childbearing women may be expected to lead to far more conservative and appropriate use of cesarean section than is now occurring… (*9*)".

Other suggested solutions are: (a) to provide access and caregivers with conservative practice style and low cesarean rates to pregnant women; (b) delay of women in labor going to hospital until labor is established; (c) a support companion for women in labor; (d) maternity care providers' retaining and applying skills to facilitate vaginal delivery, such as manually turning breech babies; (e) when possible, avoiding interventions which increase likelihood of cesarean delivery such as continuous EFM, labor induction, and early epidural; and (f) facilities limiting

cesareans to clearly established indications and taking measures to deal with unsupported indications, such as large baby, *etc*. (*8*).

8. Conclusion

Since cesarean sections generally have more medical risk than benefit, they should not be performed for non-medical reasons even before considering the enormous waste of medical and financial resources. Even if there are medical reasons for doing cesarean sections, there are limited parameters for cesarean deliveries considering a medical risk/reward analysis. The various professionals involved in maternal health should take care to see that the cesarean rate does not further increase and to lower the rate to one based on medical appropriateness. Since one primary reason for the tendency to perform cesareans has been the medicalization of the normal birth process, greater use of independent midwives and out-of hospital settings in the childbirth process is one of the possible solutions.

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Brief Report

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The expression of HER-2 in extramammary Paget's disease

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Summary **Extramammary Paget's disease (EMPD) is a rare intraepidermal adenocarcinoma. The common sites of EMPD involvement are the vulva, perineal, perianal, scrotal and penile skin. Several studies have shown that HER-2/neu, also known as c-erbB-2, is amplified and overexpressed in many cancers. In this study, we investigated the expression and clinical significance of HER-2 in Japanese patients with EMPD. Keratinocytes in epidermis were slightly positive for HER-2. As for EMPD, 19 of 31 EMPD were positive for HER-2 (61%). There is significant correlation between the presence of invasion and strong positivity (3+) for HER-2 ($p < 0.02$). Furthermore, there is significant correlation between the presence of lymph node metastasis and strong positivity (3+) for HER-2 ($p < 0.02$). These results suggest that patients with EMPD strongly positive for HER-2 may have high risk for lymph node metastasis and should be followed up carefully. The observed overexpression of HER-2 in EMPD presents a potential therapeutic target for adjuvant treatment of this disease. Treatment with trastuzumab is well established in breast cancer with HER-2 overexpression and is recommended by several consensus statements. The results of the present study indicate that targeting therapies for HER-2, such as trastuzumab, may be used for EMPD particularly in patients with invasive and/or metastatic EMPD.**

Keywords: Immunostaining, invasion, metastasis, overexpression, therapeutic target

1. Introduction

Extramammary Paget's disease (EMPD) is a rare intraepidermal adenocarcinoma with similar clinical features to inflammatory reactions. The common sites of EMPD involvement are the vulva, perineal, perianal, scrotal and penile skin. The diagnosis of EMPD is frequently delayed and there is a high incidence of associated invasive disease. EMPD presents with well-demarcated, erythematous or leucoplakic plaques. Most cases of EMPD appear eczematous but others are crusting, scaling, lichnoid, or ulcerated. Metastatic dissemination, especially to regional lymph nodes, can also occur. The diagnosis of EMPD rests on the histological identification of unique infiltrating intraepithelial neoplastic cells showing glandular differentiation. Paget's cells are large round cells with abundant pale cytoplasm and large vesicular nuclei. Mitotic figures are unusual and Paget's cells are distributed singly or in groups within the epidermis and epithelium of adnexal structures.

There are two explanations proposed for the pathogenic origin of EMPD. It is thought that Paget's cells either arise within the pore portion of an apocrine duct (*1*) or from the multipotential cells in the epidermis (*2*).

Immunohistochemical analyses have been used both to diagnose EMPD and to determine the origin of Paget's cells. Typically Paget's cells are known to be positive for apocrine and eccrine markers, such as low molecular weight cytokeratins (CK), gross cystic disease fluid protein-15 (GCDFP-15), periodic acid-Schiff (PAS), and carcinoembryonic antigen (CEA).

EMPD is histologically closely related to Paget's disease of the mammary gland, which is an adenocarcinoma affecting the skin of the nipple. Several studies have shown that HER-2/neu, also known as c-erbB-2, is amplified and overexpressed in almost all cases of mammary Paget's disease, in contrast to about 20% of invasive ductal breast carcinomas (*3,4*).

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HER-2 overexpression in breast cancer is correlated with a more aggressive behavior. Previous studies have investigated the expression of HER-2 in EMPD (*4-7*). However, inconsistent results and lack of a large number of cases have resulted in different outcomes among studies.

The HER-2 gene, located on chromosome 17q21, encodes for the 185-kD transmembrane glycoprotein growth factor receptor processing a tyrosine kinase domain (*8,9*). The HER-2 protein recognizes growth stimuli and acts by 2 principal pathways, phosphatidylinositol 3 kinase (PI3K) and extracellular signal-regulated kinase (ERK). Activation of both pathways is intimately correlated with aggressiveness in various cancers (*10,11*). In many human cancers, the HER-2 oncogene is involved in transformation and progression of many human cancer cells (*12*). HER-2 gene amplification and/or HER-2 protein overexpression are common in many human cancers, such as breast, ovarian, and gastric adenocarcinoma (*12).*

In this study, we investigated the expression and clinical significance of HER-2, PAS, CEA, CK7 and epithelial memebrane antigen (EMA) in Japanese patients with EMPD.

2. Materials and Methods

2.1. *Patients*

Besides 5 normal skin samples, skin samples were obtained from 31 patients with EMPD. Institutional review board approval and written informed consent were obtained according to the Declaration of Helsinki. All patients with EMPD were diagnosed by clinical and histopathological findings. All samples were fixed in neutral buffered formalin, embedded in paraffin, and prepared for hematoxylin-eosin examination.

2.2. *Immunohistochemical stainings*

Immunohistochemical staining on paraffin-embedded sections was performed using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's recommendations. Serial 4 μm thick sections were mounted on silane-coated slides (Dako), then deparaffinized with xylene and rehydrated through a graded series of ethyl alcohol and PBS. The sections were then incubated with various antibodies overnight at 4°C. Antibodies against HER-2 (CB11, NCL; 1:50), CEA (A0115, Dako; 1:2,000), EMA (M0613, Dako; 1:200), and CK7 (M7018, Dako; 1:50) were used for primary staining. The immunoreactivity was visualized with Vector Red (Vector Labotatories). The sections were then counterstained with hematoxylin. We used the following grading system: 1+ for slight staining, 3+ for strong staining, and 2+ for staining between 1+ and 3+.

2.3. *Statistical analysis*

Statistical analysis was carried out with Fisher's exact probability test for the analysis of frequency. Two-tailed *p* values less than 0.05 were considered significant.

3. Results

3.1. *Immunoreactivity of PAS, CEA, CK7, and EMA with EMPD cells*

First, we investigated the immunoreactivity of PAS, CEA, CK7 and EMA with EMPD cells. The EMPD cells from all the patients included in this study were positive for PAS, CEA, CK7 and EMA (Figure 1). These results suggested that PAS, CEA, CK7 and EMA stainings were all useful for diagnosis of EMPD, but not for disease activity or severity.

3.2. *Immunoreactivity of HER-2 with EMPD cells*

We investigated the immunoreactivity of HER-2 with normal skin samples. Keratinocytes in epidermis were slightly positive for HER-2 (data not shown). As for EMPD, 19 of 31 EMPD were positive for HER-2 (61%, Table 1 and Figure 2). There is a significant correlation between the presence of invasion and strong positivity (3+) for HER-2 ($p < 0.02$). Furthermore, there is a significant correlation between the presence of lymph node metastasis and strong positivity (3+) for HER-2 ($p < 0.02$). However, there is no significant correlation between the presence of invasion and positivity (1+, 2+, or 3+) for HER-2, or between the presence of lymph node metastasis and positivity (1+, 2+, or 3+) for HER-2.

4. Discussion

The HER-2 gene, located on chromosome 17q21, encodes for the 185-kD transmembrane glycoprotein growth factor receptor processing a tyrosine kinase domain (*8,9*). The HER-2 protein recognizes growth stimuli and acts by 2 principal pathways, phosphatidylinositol 3 kinase (PI3K) and extracellular signal-regulated kinase (ERK). Activation of both pathways is intimately correlated with aggressiveness in various cancers (*10,11*).

In many human cancers, the HER-2 oncogene is involved in transformation and progression of many human cancer cells (*12*). HER-2 gene amplification and/or HER-2 protein overexpression are common in many human cancers, such as breast, ovarian, and gastric adenocarcinoma (*12*).

It is generally agreed that overexpression of proto-oncogenes including myc and HER-2 occurs late in the progression of many human tumors (*13,14*). In this study, there was significant correlation between the presence of invasion and strong positivity (3+)

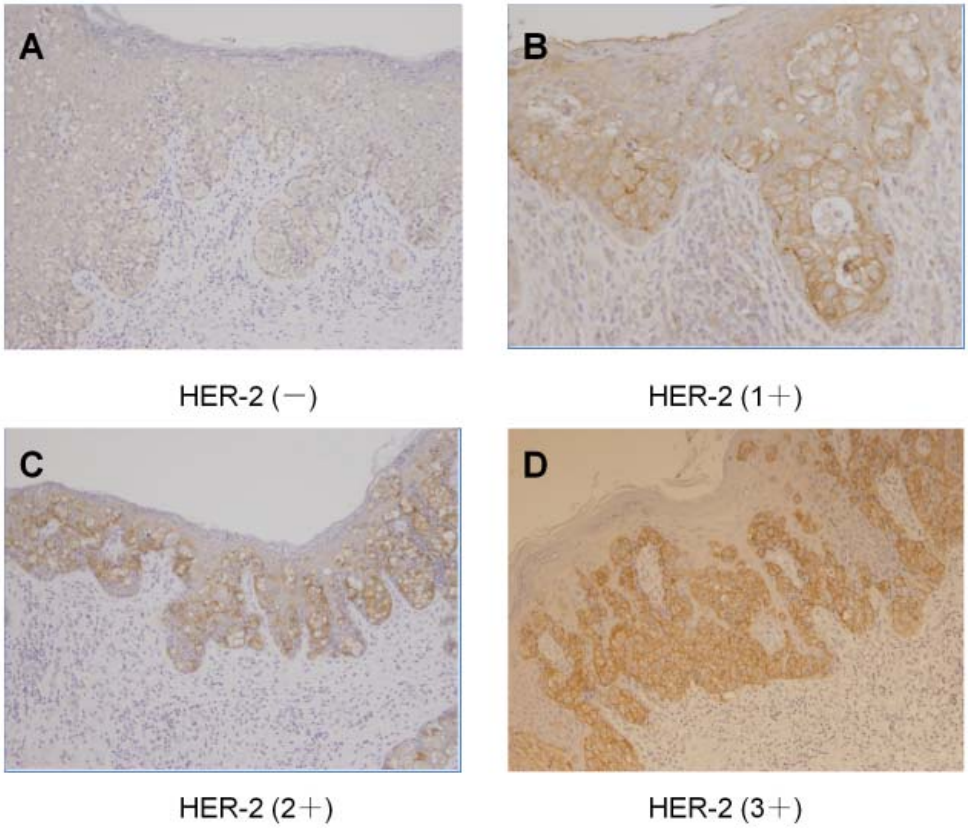

Figure 1. Immunohistochemical staining of HER-2 in extramammary Paget's disease cells. The membrane of tumor cells are clearly stained. **(A)** HER-2 (-); **(B)** HER-2 (1+); **(C)** HER-2 (2+); **(D)** HER-2 (3+).

Table 1. Immunohistochemical staining of HER-2 in extramammary Paget's disease

Items	HER-2				*p* Values
	-	1+	2+	3+	
Age (from 48 to 93)					
Sex					$p < 0.337$
M ($n = 18$)	6	8	2	2	
F ($n = 13$)	6	6	1	0	
Location					$p < 0.128$
Vulva ($n = 13$)	6	6	1	0	
Scrotum ($n = 16$)	4	8	2	2	
Perianal ($n = 2$)	2	0	0	0	
Ca. or *in situ*					$p < 0.02$
Ca. ($n = 4$)	0	2	0	2	
in situ ($n = 20$)	11	6	3	0	
Microinvasion ($n = 7$)	1	6	0	0	
CK7					
+ ($n = 26$)	12	11	1	2	
PAS					
+ ($n = 19$)	7	8	3	1	
CEA					
+ ($n = 27$)	11	12	3	1	
EMA					
+ ($n = 21$)	10	9	1	1	
LN meta. ($n = 4$)	0	2	0	2	$p < 0.02$
Death ($n = 1$)	0	0	0	1	

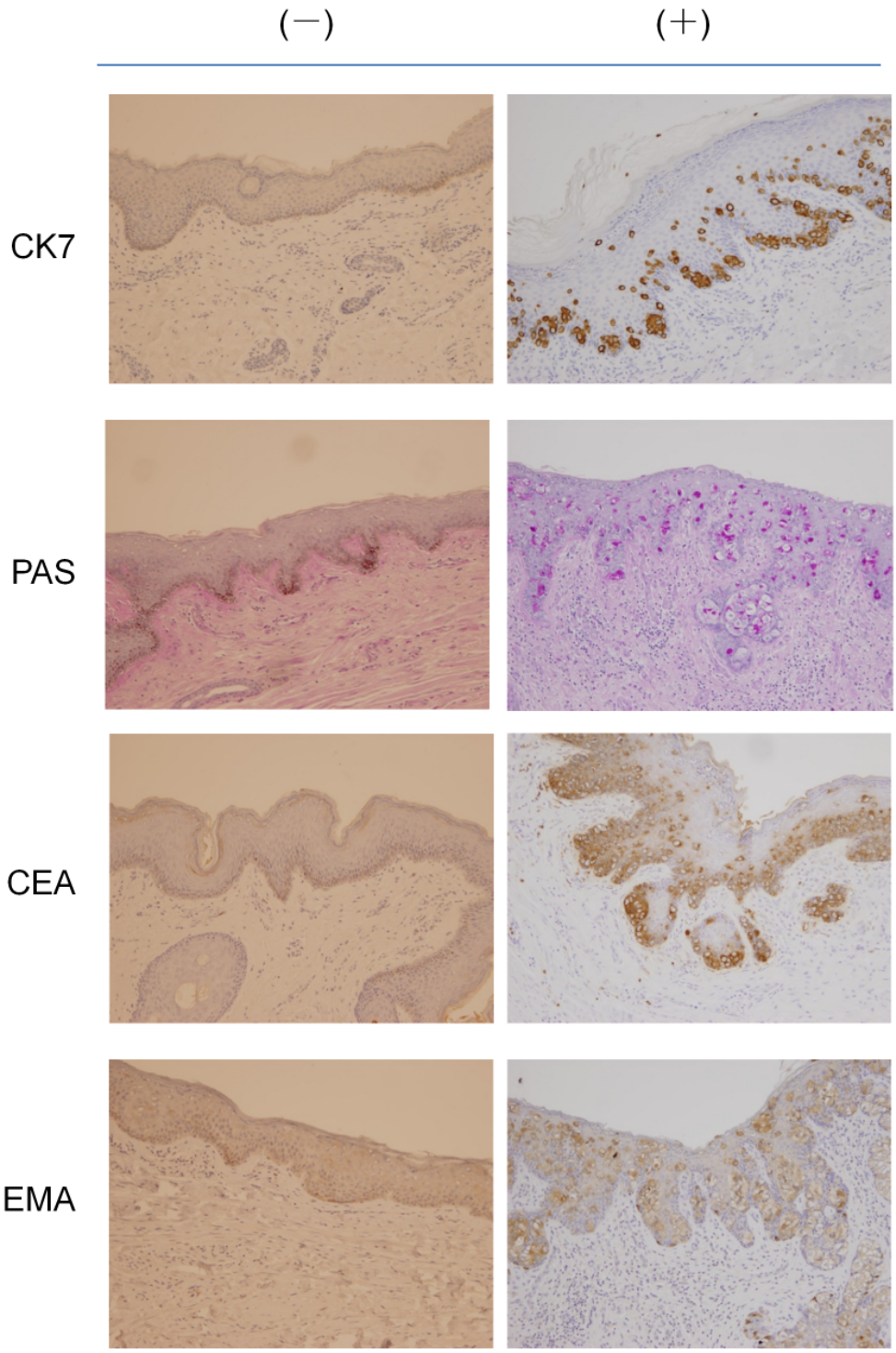

Figure 2. Immunohistochemical staining of cytokeratin 7 (CK7), periodic acid-Schiff (PAS), carcinoembryonic antigen (CEA), epithelial membrane antigen (EMA) in extramammary Paget's disease (EMPD) cells.

for HER-2 ($p < 0.02$). Furthermore, there was a significant correlation between the presence of lymph node metastasis and strong positivity (3+) for HER-2 ($p < 0.02$). This indicates that HER-2 overexpression plays a crucial role in the invasion and lymph node metastasis of EMPD. This observation is similar to several breast cancer studies showing enhancement of metastatic potential by HER-2 overexpression (*15*), the demonstration of potentially metastatic cell subpopulations expressing HER-2 within the individual cancer tissue (*16*), and correlation with HER-2 overexpression and random cell migration (*17*). These results suggest that patients with EMPD strongly positive for HER-2 may have a high risk of lymph node metastasis and should be followed up carefully.

As described above, HER-2 overexpression in breast cancer is correlated with more aggressive behavior. However, inconsistent results and lack of

a large number of cases have resulted in different outcomes among previous studies investigating the expression of HER-2 in EMPD (*4-7*). This is probably because of a small number of patients included in the previous studies. There is also another possibility that previous studies included a small number of patients with invasion or those with lymph node metastasis.

The observed overexpression of HER-2 in EMPD presents a potential therapeutic target for adjuvant treatment of this disease. Treatment with trastuzumab is well established in breast cancer with HER-2 overexpression and is recommended by several consensus statements (*18*). The results of the present study indicate that targeting therapies for HER-2, such as trastuzumab, may be used for EMPD, particularly in patients with invasive and/or metastatic EMPD.

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Brief Report

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Once-daily tacrolimus in living donor liver transplant recipients

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Summary **Once-daily tacrolimus (denoted here simply as OD) is a recently developed extended-release drug formulation. The purpose of the present study was to pharmacokinetically evaluate tacrolimus exposure and determine the feasibility of its de novo use in liver transplant recipients in the perioperative period. This was an open-label, single center study. Eligible patients were 18 to 65 years of age in the perioperative period after a liver transplant. Patients were initially treated with intravenous tacrolimus and then converted to the 10× milligram-for-milligram daily dose of OD administered once daily. Twenty-four-hour pharmacokinetic profiles were obtained on day 7 after the conversion. Laboratory and safety parameters were also evaluated. A total of 9 patients received OD, were successfully converted, and provided pharmacokinetic profiles. Intravenous tacrolimus and OD resulted in similar areas under the curve for 24 h ($AUC_{0\text{-}24}$) of tacrolimus. OD was well tolerated with a safety profile comparable to that of intravenous tacrolimus. The $AUC_{0\text{-}24}$ correlated with the minimum concentration of OD (R = 0.49). Renal and liver functions remained stable. None of the patients experienced acute rejection during the observation period. OD and intravenous tacrolimus provide equivalent drug exposure, allowing conversion of selected liver transplant recipients from intravenous tacrolimus to OD in the peri-operative period.**

Keywords: Once-daily, donor, living donor liver transplantation, tacrolimus

1. Introduction

A twice-a-day tacrolimus formulation (TAC, Prograf®, Astellas Pharmaceutical Corporation, Tokyo, Japan) is commonly used to prevent organ rejection in allogeneic kidney, liver, and heart transplant recipients (*1*). Medication compliance after transplantation, however, is a serious problem (*2*); a once-daily regimen could potentially improve compliance while maintaining safety. A once-daily tacrolimus formulation (OD) (*3*) that has the similar level of absorption as TAC with a reduced peak was recently developed and might help to resolve problems with compliance.

The present study reports the pharmacokinetics of tacrolimus during conversion from intravenous tacrolimus to OD in liver transplant recipients in the perioperative period. The aim of the present study was to determine the safety and tolerability of OD in these patients.

2. Patients and Methods

From February 2009 to May 2010, this institution performed 12 living donor liver transplants (LDLTs) and all of the patients involved were enrolled in this study. The recipients consisted of 5 men and 7 women with a median age of 49 years (range: 37-61 years). The median Model for End-stage Liver Disease score was 16 (range: 3-33). LDLT was indicated in cases of virus-related cirrhosis with or without hepatocellular carcinoma (n = 9) and cholestatic disease (n = 3).

The surgical technique for LDLT and the process

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of donor selection and evaluation have been described elsewhere (*4*,*5*). Similar to the majority of liver transplantation centers worldwide (*6*), this institution uses a tacrolimus-based immunosuppression regimen. All of patients included in the present study initially received the same immunosuppressive regimen with TAC and methylprednisolone (*7*). In brief, TAC was administered by continuous intravenous infusion at a dose of 2.5 ug/kg/h just after surgery. After the whole blood level of tacrolimus reached 17-18 ng/mL, the dose was adjusted so that this level was maintained during the first week after surgery. Intravenous methylprednisolone was started during surgery (20 mg/kg/day) and gradually tapered afterwards. When gastrointestinal function returned, OD and steroids were given orally. Steroid treatment was not discontinued in any of the patients.

Serial collection of whole blood samples to evaluate the pharmacokinetic profiles was performed at 0 (pre-dose), 1, 2, 3, 5, 10, and 24 h on day 7 after conversion to OD. On the other days, blood samples were obtained every 12 hours. Whole blood samples for pharmacokinetic analysis were frozen (−20°C) until they were shipped to a central facility, where they were analyzed using a validated liquid chromatography/ tandem mass spectrometry assay (lower limit of quantification 0.1 ng/mL). Whole blood tacrolimus concentrations below the lower limit of quantification were assigned a value of zero for calculation of the derived pharmacokinetic parameters. The area under the curve of the blood concentration-time curve (ng × h/mL) from 0 to 24 h (AUC_{0-24}) after dosing was calculated using the linear trapezoidal rule. Minimum concentrations (C_{min}; ng, trough) were determined based on the observed whole blood tacrolimus concentration at 24 h on days when pharmacokinetic profiles were obtained. Total clearance (CL_{tot}), oral clearance (CL_o), and bioavailability (BA) were calculated as follows:

CL_{tot} = Doses of intravenous tacrolimus/AUC (l/h)
CL_o = Doses of OD/AUC (l/h)
BA = CL_{tot}/CL_o (%)

All adverse events and all serious adverse events were recorded during the pharmacokinetic study. Rejection episodes, all serious adverse events, and the following protocol-defined adverse events were recorded: post-transplant diabetes mellitus, hyperlipidemia, hypertension, infection (viral, bacterial, and fungal), renal dysfunction (serum creatinine level ≥ 2.0 mg/dL), hepatic dysfunction, tremor, malignancy, and adverse events leading to study drug dose changes or study drug discontinuation. The incidence of biopsy-confirmed acute rejection and patient and graft survival were assessed throughout the study. Safety was assessed based on the incidence of adverse events and the results of routine clinical laboratory tests and recorded vital sign measurements.

Statistical analysis was performed using JMP 8.2 computer software (SAS Inc., Cary, NC). JMP 5.1 was used to perform one-way analysis of variance and *t*-tests. *P* values of less than 0.05 were considered statistically significant for *t*-tests and *p* values of less than 0.01 were considered statistically significant for analysis of variance with Bonferroni's correction (in comparison to preoperative levels).

3. Results and Discussion

In three patients, OD was started 10, 12, and 18 days after LDLT, but the trough level did not reach the target level and the study was stopped.

In the remaining 9 patients, intravenous tacrolimus was successfully converted to OD for an average of 9 days (range: 7-20 days) after LDLT. The dose of intravenous tacrolimus just before the conversion was 9.6×10^{-3} mg/kg/day (0.64 mg/day). The blood concentration of tacrolimus after conversion and the pharmacokinetic parameters are shown in Figure 1 and Table 1. Intravenous tacrolimus and OD resulted in a similar AUC_{0-24} of tacrolimus. OD was well tolerated with a safety profile comparable to that of intravenous tacrolimus. The AUC_{0-24} correlated with the C_{min} for OD ($R = 0.49$) (Figure 2). Renal and liver functions remained stable. None of the patients experienced acute rejection during the observation period.

There were no adverse effects (post-transplant diabetes mellitus, hyperlipidemia, hypertension, renal dysfunction, or hepatic dysfunction) or acute rejection episodes during the observation period.

Overall, OD was well tolerated in this study and the adverse event profile was consistent with that of oral tacrolimus. In addition, serum creatinine levels remained stable after conversion to OD. There were no new cases of posttransplant diabetes or glucose intolerance and there was no increase in adverse events associated with tacrolimus use after conversion to OD. None of the patients experienced a biopsy-confirmed acute rejection episode during the pharmacokinetic study. Finally, there were no changes in the use of concomitant medication, including adjunctive immunosuppressive agents, during this study.

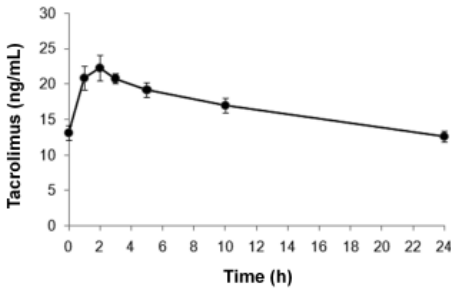

Figure 1. Blood concentration of tacrolimus after conversion.

Table 1. Pharmacokinetic parameters (n = 9)

	Intravenous tacrolimus	OD
Dose/BW (mg/kg)	0.0105 ± 0.0021	0.124 ± 0.047
C_{min}/Dose/BW ([ng/mL]/[mg/kg])	1,468 ± 321	118 ± 57
C_{max}/Dose/BW ([ng/mL]/[mg/kg])	-	223 ± 80
$AUC_{0\text{-}24}$ ([ng∗h/mL]/[mg/kg])	35,232 ± 7,702	3,706 ± 1,635
Clearance/BW (mL/h/kg)	CL_{tot} = 29.7 ± 7.1	CL_o = 316 ± 128
Bioavailability (%)	10.6 ± 4.0	-

OD, once-daily tacrolimus formulation; BW, body weight; $AUC_{0\text{-}24}$, area under the blood concentration-time curve from 0 to 24 h after dosing was calculated by the linear trapezoidal rule; C_{min}, minimum concentration (trough) values; $C_{max,}$ maximum concentration; $CL_{tot,}$ total clearance; CL_o, oral clearance.

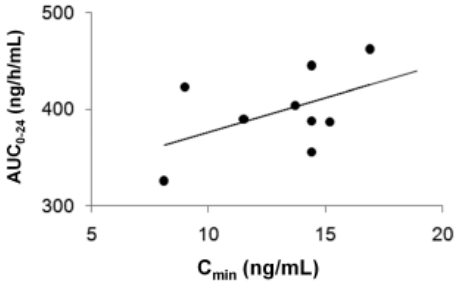

Figure 2. Correlation between AUC and C_{min}. Correlation was expressed as AUC = 7.1018 × C_{min} + 305.09. (R^2 = 0.24, p = 0.17)

The level of steady-state intravenous tacrolimus exposure and 10× dose of OD were equivalent in perioperative liver transplant recipients regardless of sex or the presence of diabetes at the time of transplantation. The high correlation of exposure to trough levels for OD indicates that the system for monitoring TAC can be effectively used with patients receiving OD. There was significantly less intra-subject variability in tacrolimus exposure after conversion to OD.

The new TAC formulation provides a convenient, once-daily dosing option (*8*). Therapeutic regimens for transplant recipients are often complex and thus contribute to a high incidence of medication noncompliance and increased mortality and morbidity, including late acute rejection, late graft loss, and development of chronic rejection. Compliance is reported to increase from 59% with three-times-a-day dosing regimen to 83% with once-daily dosing regimens, suggesting that administering fewer doses is a simple and effective way to improve compliance (*9*). The introduction of a once-daily dosing formulation may prove to be a valuable addition to the treatment armamentarium for transplant recipients.

4. Conclusion

In conclusion, liver transplant recipients in the perioperative period can be safely converted from intravenous tacrolimus to a 10× milligram-for-milligram daily dose of OD in the morning. A once-daily dosing regimen of tacrolimus can improve patient compliance while maintaining effective immunosuppression.

Acknowledgements

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Brief Report

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The advantage of using IS6110-PCR *vs*. BACTEC culture for rapid detection of *Mycobacterium tuberculosis* from pleural fluid in northern India

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Summary **Pleural tuberculosis is an extra-pulmonary disease which poses a diagnostic dilemma. The detection of mycobacterial DNA by IS6110 polymerase chain reaction (PCR) in clinical samples is a promising approach for the rapid diagnosis of pleural tuberculosis infections. The aim of the present study is to evaluate the advantage of using IS6110 PCR for rapid detection of *Mycobacterium tuberculosis* (*M. tuberculosis*) from pleural fluid. 102 clinically suspected cases of pleural tuberculosis cases were enrolled from inwards and outwards of the Department of Pulmonary Medicine at Chattrapati Shahuji Maharaj Medical University, Lucknow from April 2007 to April 2010. The pleural fluids were processed at the Mycobacteriology Laboratory of Department of Microbiology at Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, Pleural fluid samples were processed and examined by Ziehl Neelsen (ZN) staining for acid fast bacilli and detection of *M. tuberculosis* by BACTEC culture. We applied IS6110 PCR to detect specific *M. tuberculosis* complex in pleural fluid samples. We found a significant difference in sensitivity of different tests, acid fast bacilli were detected in 17 (16.6%) samples by ZN Staining , 47 (46.1%) by BACTEC culture and using IS6110 PCR, 62 (60.7%) were positive for IS6110 PCR for *M. tuberculosis*. We found IS6110 PCR was much more sensitive than ZN staining and BACTEC culture. IS6110 PCR detection of *M. tuberculosis* may be very useful in cases that are highly suspect as pleural tuberculosis and those that are negative for AFB and culture. IS6110 PCR may gain an immense prospective to better clinicians ability to improve diagnosis of pleural tuberculosis.**

Keywords: Tuberculosis, pleural fluids, *Mycobacterium tuberculosis (M. tuberculosis)*, polymerase chain reaction

1. Introduction

Pleural tuberculosis is responsible for 30-80% of all pleural effusions encountered and may complicate tuberculosis in 31% of all cases (*1*). Thus, tuberculous pleuritis remains a major contributor to worldwide morbidity and mortality. It poses diagnostic and therapeutic problems due to the low sensitivity of the diagnostic tools. Conventional culture is time consuming and lacks sensitivity; smears for acid-fast bacilli (AFB) is rapid but the sensitivity has not been evaluated in pleural fluid. However, it has been reported to be positive in less than 10-37% of patients and mycobacterial cultures in variable proportions (12-80%) in different body fluids (*2*). The diagnostic dilemma can affect treatment by either delaying it or causing inappropriate empiric therapy for tuberculosis (TB) to subjects without mycobacterial infections or

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with atypical mycobacteria (*3*). The development of a diagnostic method capable of rapidly identifying *Mycobacterium tuberculosis* (*M. tuberculosis*) in pleural fluid from patients remains a worthwhile aim. Several studies have been reported on PCR to detect *M. tuberculosis* (*4-8*). The detection of the IS6110 insertion element present in multiple copies (*9*) can be used to detect *M. tuberculosis* complex, but no other mycobacterial species. The aim of the present study is to evaluate the advantage of IS6110 PCR *vs*. BACTEC culture for rapid detection of *M. tuberculosis* from pleural fluid in Northern India.

2. Materials and Methods

2.1. *Study design*

The study was performed prospectively in a blinded manner. Study setting was Referral Medical Institutions in Northern India.

2.2. *Study population*

A total of 102 clinically suspected cases of pleural tuberculosis were enrolled from inwards and outwards of the Department of Pulmonary Medicine, Chhatrapati Shahuji Maharaj Medical University, Lucknow, from April 2007 to April 2010.

2.3. *Clinical information and data collection*

The clinical history regarding disease, present and past history of TB and anti-tuberculosis treatment (ATT) taken along with information regarding family history of tuberculosis was obtained in prescribed proforma. Clinical examination and pleural fluid (approximately 2-5 mL) was aspirated, after informed consent, by the clinician and stored at 4°C and transferred to the Mycobacteriology Section of Department of Microbiology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, India (within 4 h of collection). Patient profiles and clinical data were retrieved from the medical record case file.

2.4. *Processing and microbiological test of pleural fluid*

Pleural aspirate was divided into two parts, one part was kept at −20ºC for PCR processing and the other part was processed for mycobacterial smear preparation and BACTEC culture. Smears were stained using the ZN method and examined for AFB (*10*). BACTEC vials were incubated and interpreted as per Becton Dickinson (BD, Sparks, MD, USA) manual instructions (*11*). The p-nitro-α-acetylamino-β-hydroxy propiophenone (NAP) identification was done to differentiate *M. tuberculosis* complex from non tuberculous mycobacteria (*11*).

2.5. *Extraction of DNA*

Extraction of DNA was done by the CTAB (cetyl-tri-methyl-ammonium bromide) -phenol chloroform extraction method (*12*). First the pleural aspirate was centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and the pellet suspended in 567 µL of TE (Tris EDTA, pH 7.4) buffer, 30 µL 10% SDS (sodium dodecyl sulfate) and 3 µL proteinase K (20 mg/mL), mixed and incubated at 37°C for 1 h. After incubation, 100 µL of 5 M NaCl and 80 µL of high-salt CTAB buffer (containing 4 M NaCl), 1.8% CTAB was added and mixed followed by incubation at 65°C for 10 min. An approximate equal volume (0.7-0.8 µL) of chloroform-isoamyl alcohol (24:1) was added, mixed thoroughly and centrifuged for 4-5 min in a microcentrifuge at 12,000 rpm. The aqueous viscous supernatant was carefully decanted and transferred to a new tube. An equal volume of phenol: chloroform-isoamyl alcohol (1:1) was added followed by a 5 min spin at 12,000 rpm. The supernatant was separated and then mixed with 0.6 volume of isopropanol to get a precipitate. The precipitated nucleic acids were washed with 75% ethanol, dried and re-suspended in 100 µL of TE buffer.

2.6. *Primers and PCR*

The amplification reaction was performed in a final volume of 20 µL. The reaction mixture contained 10 µL Pyrostart Fast PCR Master Mix 2X (dNTP, Taq polymerase with $MgCl_2$, Fermentas, India), 1 µL (10 pmole) of each primer, 3 µL water (nuclease free) and 5 µL of extracted DNA. The oligonucleotide primers (*13*) used were P1 and P2, and are: 5'-CCT GCG AGC GTA GGC GTC GG3' and 5' CTC GTC CAG CGC CGC TTC GG 3' respectively (SBS Gentech Co., Ltd.). These primers amplified a target fragment 123 base pairs (bp) from the insertion of the *M. tuberculosis* sequence element IS6110. The PCR amplification was done in a thermal cycler (MJ Research ,PTC-100, GMI, Inc, USA), which involved 40 cycles of denaturation at 94°C for 2 min, annealing of primers at 68°C for 2 min, and primer extension at 72°C for 1 min. The amplified products were separated on 2% agarose gels and visualized on a UV-light transilluminator (Bangalore Genei, Bangalore, India). The presence of the 123 bp fragment indicated a positive test (*M. tuberculosis* complex). The positive controls included the DNA of the H37Rv strain. Negative control included PCR grade water.

2.7. *Statistical analysis*

We assumed statistical significance at $p < 0.05$. The sensitivity, specificity, positive predictive value and negative predictive values were calculated with a 95% confidence interval (95% CI) using the standard formulas

considering BACTEC as the gold standard (*14*).

3. Results and Discussion

3.1. *Patients characterization*

A total of 102 patients were enrolled in our study. Of these 102 patients, 77 (75%) patients were males and 25 (25%) were females. The mean age of all patients was 30.4 ± 13.2 years. Patients 25-44 years of age accounted for 42.2% of the total cases. Among all cases, 70 were newly detected cases (68.6%), 25 were previous treated cases (24.5%), 5 were on treatment (4.9%) and 2 were unknown (1.9%). The history of contact with TB patients was determined in 20 cases (19.6%), 19(18.2%) had a history of diabetic mellitus and a past history was present in 11 (10.7%). In the case of HIV presentation, 2 cases were HIV positive (2.5%) and they had an antiretroviral therapy (ART) and ATT taken and 100 were HIV negative (97.5%). We found common symptoms of pleural tuberculosis, 17 patients had hempotosis (16.6%), 62 cough (60.7%), 75 fever (75.9%), 71 anorexia and/or weight loss (70%), 53 chest pain (51.9%), 58 night sweat and or chills (56.5%) and 21 dyspnoea (20.5%).

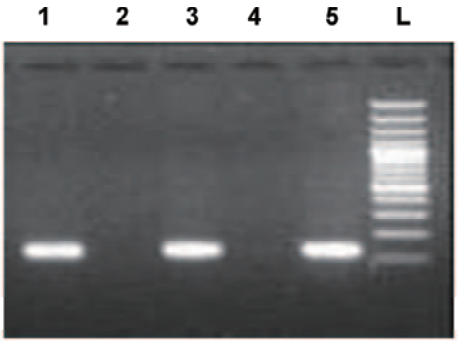

Figure 1. Gel documentation of electrophoresis separation of the amplicon into 2% agarose gel is documented across Lanes 1-5. The presence of a 123 bp. Amplicon in the **Lanes 1** and **3** indicated the presence of the target while the absence of the amplicon in the **Lane 2** pointed towards the absence of the target. **Lane 4** was Negative Control (PCR water) and **Lane 5** was Positive Control (H37Rv). **Lane L** was 100 bp Ladder well shown.

3.2. *Detection rate of M. tuberculosis by smear, BACTEC culture and IS6110 PCR*

All pleural fluid samples in the suspected cases of pleural TB were found to be AFB positive in 17 (16.6%). The sensitivity of AFB staining on pleural fluid was 16.6 % and its detection rate for *M. tuberculosis* by AFB staining was 16.6%. The detection of *M. tuberculosis* by BACTEC culture was 47 (46.1%). Using IS6110 PCR, 62 (60.7%) were positive for IS6110 PCR for *M. tuberculosis*, and results are shown (Figure 1).

3.3. *Comparison of sensitivity of PCR test vis-a-vis other tests*

Seventeen patients were positive with AFB and PCR was positive (100%) for *M. tuberculosis*, 85 patients were negative with AFB and PCR was positive for 45 (52.9%) but culture was subsequently positive in 32 (31.3%) patients. The sensitivity of PCR testing was 100% for 15 patients positive for both AFB and culture, where we found low sensitivity for 1 (50%) of 2 patients were AFB positive with negative cultures. 32 patients (93.7%) had PCR sensitivity with smears negative and cultures positive. In other words, 30 of the 32 *M. tuberculosis* complex culture positives were positive for IS6110 sequences. Therefore, given the sensitivity of 93.7 % and 53 negatives by all other tests used (smear negative and culture negative) samples which were positive by PCR were 16 (30.1%). These were not likely to represent false positive results because repeated PCR tests were positive for these samples and these samples belonged to highly suspected cases of pleural tuberculosis which responded to antitubercular treatment (Table 1).

3.4. *Comparison of sensitivity and specificity of PCR tests and smear microscopy vs. BACTEC as the gold standard*

On taking BACTEC culture as the gold standard the sensitivity, specificity, positive predictive value and negative predictive value of microscopy and PCR are given in Table 2. The sensitivity, specificity, positive

Table 1. Comparison of sensitivity of PCR test via a via others tests

Test	No. (%)	PCR Results (*n*)		Sensitivity of PCR test (%)
		Positive	Negative	
Smear positive	17 (16.6)	17	0	100
Smear negative	85 (83.4%)	45	40	52.9
BACTEC Positive	47 (46.1)	45	2	95.7
BACTEC negative	55 (53.9)	17	38	30.9
Smear positive BACTEC positive	15 (14.8)	15	0	100
Smear Negative BACTEC Positive	32 (31.4)	30	2	93.7
Smear Positive BACTEC Negative	2 (1.9)	1	1	50
Smear Negative and BACTEC negative	53 (51.9)	16	37	30.1

Table 2. Comparison of sensitivity and specificity of PCR test , smear microscopy and BACTEC culture in 102 patients

Test	BACTEC Culture *M. tuberculosis*		Sensitivity	Specificity	PPV	NPV
	Positive	Negative				
PCR						
Positive	45	17	95.7 %	69.1 %	72.5%	95.0%
Negative	2	38				
Smear						
Positive	15	2	31.9%	96.3 %	88.2 %	62.3 %
Negative	33	52				

Sensitivity, specificity, positive and negative predictive values of PCR and microscopy were calculated using BACTEC culture results as the gold standard. PPV: positive predictive value; NPV: negative predictive value.

predictive value and negative predictive values were calculated with a 95% confidence interval (95% CI) using standard formulas. Among 102 patients, 62 were positive with IS6110 PCR and 17 were positive with AFB smear microscopy. By defining BACTEC culture as the gold standard for comparative usefulness of the PCR assay, the sensitivity and specificity of the assay were 95.7% (95% CI 0.85-0.98) and 69.1% (95% CI 0.55-0.79) shown in Table 2. We found that sensitivity of PCR (95.7%) was very high in comparison to smear microscopy but specificity of PCR (68.5%) was lower than smear microscopy (96.3%). The variation in the specificity of IS6110 PCR may be due to the varied methods of extraction of DNA, use of different sets of PCR primers designed to amplify IS6110 nucleotides and expertise in performing the PCR technique.

Pleural tuberculosis is a major, treatable cause of exudative pleural effusion. Chakrabati *et al*. (2006) stated that the epidemiology and demographics of tuberculous pleurisy are changing due to the impact of HIV co-infection and the increasing amount of pleural effusion seen as part of reactive diseases (*15*). The diagnosis of pleural fluid is still a challenge for number of reasons. The lack of adequate sample volumes and the non uniform distribution of microorganisms contribute to this problem. Escudero BC *et al.* (1990) suggested that the diagnosis of TB pleurisy is usually accomplished using radiological and clinical findings, pathology of pleural tissue from biopsy, and several laboratory methods (*16*). Conventional methods include direct examination of pleural fluid with ZN staining of acid-fast bacilli and culture. ZN staining is rapid and inexpensive but requires a bacilli concentration of 10,000/mL and has a low sensitivity of approximately 0-1% (*16*). Earlier studies suggested that culture was more sensitive (11-50%); where only 10-100 bacilli yield the diagnosis, but required 2-6 weeks to grow *M. tuberculosis* (*17*). Pleural biopsy studies have high sensitivity (70-80%), but the procedure is not free of risk (*18,19*). At the present time, the most reliable method for diagnosis of pleural TB is the detection of *M. tuberculosis* in pleural specimens. Rapid diagnosis of pleural TB is critical in order to reduce morbidity and mortality. Study data showed PCR was a molecular biology technique that can detect *M. tuberculosis* genome in pleural fluid or tissue specimens (*20,21*). The sensitivity (31.3-81%) and specificity (96.6-100%) were variable. Studies conducted by Parandaman V *et al*. (2000), Tan J *et al*. (1995), Takagi N *et al*. (1998) and Jatana SK *et al*. (2000) showed disparate results where sensitivity is 100% and specificity varied from 70-90% (*22-25*). But these studies have been carried out on a small sample size; all of them have targeted the IS6110 insertion sequences and have engaged different sets of primers. The IS6110 PCR protocol used to detect the IS6110 insertion sequence was more sensitive than other PCR protocols (*26*). Previous studies found the IS6110 sequence present in 10-15 copies in each mycobacterial genome (*9,27*), and increased sensitivity afforded by the detection of the IS6110 insertion sequence considerably improves the yield for the detection of mycobacterial DNA in pleural fluid. Because this insertion sequence is present only in mycobacteria of the *M. tuberculosis* complex, positive results were not observed for other species of mycobacteria (*9*). Our study results suggest that IS6110 PCR is more sensitive than conventional methods, but still not absolute to identify all cases. In cases where ZN positive samples were found IS6110 PCR amplification was positive. Studies revealed that the insertion element of IS6110 primers for detection of TB, PCR directed towards the IS6110 sequence of *M. tuberculosis* have been evaluated by Villegas *et al*. (2000) (*28*). Parandaman *et al.* (2000) found 100% positive PCR of positive culture samples, but PCR was positive in 30-60% of culture negative pleural fluids (*22*). Our results have shown that PCR was positive in 15/15 (100%) from positive cultures but PCR was positive in 17/55 (30.9%) from negative cultures. Reechaipichitkul *et al*. (2000) revealed that the sensitivity and specificity of pleural fluids on culture were 17% but we found sensitivity of pleural fluid on culture were 46.1% (*29*).

In conclusion, IS6110 PCR assays can successfully be used to detect *M. tuberculosis* DNA in pleural fluid samples for a more rapid, specific and reliable TB diagnosis than the BACTEC culture and ZN staining methods. IS6110 based PCR detection of *M.*

tuberculosis may be very useful in cases that are highly suspect for pleural TB and those that are negative for AFB and culture, and it may better clinicians ability to improve diagnosis in pleural TB.

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Apolipoprotein A5 polymorphisms and risk of coronary artery disease: A meta-analysis

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Summary **The relation has not been reported consistently between the polymorphisms in the gene of apolipoprotein A5 (APO A5) and coronary artery disease (CAD). To clarify the discrepancy, we conducted a comprehensive search of PubMed and EMBASE for all available case-control studies to explore the association between two APO A5 polymorphisms and CAD. Two reviewers independently selected studies. Statistical analyses were carried out using the STATA software package v 10.0. Thirteen studies investigated the association between the APO A5 -1131T>C polymorphism and risk of CAD were selected in this meta-analysis with 5,050 cases and 7,272 controls. For the S19W APO A5 gene polymorphism, 5 studies were included with 2,196 cases and 3,933 controls. We observed a significant statistical association between Apo A5 -1131T>C polymorphism and CAD (recessive genetic model: OR = 1.73, 95% CI = 1.37-2.19; dominant genetic model: OR = 1.42, 95% CI = 1.25-1.61; allelic contrast: OR = 1.31, 95% CI = 1.22-1.39, respectively). After restricting our analysis to Chinese individuals, we found that the association was stronger. We also observed strong association between the APO A5 S19>W polymorphism and risk of CAD under a recessive genetic model. This meta-analysis reveals that the minor allele of the -1131T>C polymorphism in the promoter of APO A5 gene significantly increases the susceptibility to CAD. This effect is more pronounced in Chinese subjects.**

Keywords: Meta-analysis, gene polymorphism, coronary artery disease, apolipoprotein A5

1. Introduction

Coronary artery disease (CAD) is the leading cause of death and disability, which is believed to have a multifactorial genetic basis involving a number of genes and environmental factors that interact to contribute towards individual susceptibility (*1*). Epidemiological and clinical studies have shown that increased triglyceride (TG) concentrations are an independent risk factor of CAD (*2,3*). TG levels may be altered by a variety of genetic and environmental factors, and twin studies have also shown a strong genetic contribution to TG levels (*4*). Apolipoprotein gene cluster APOA1/C3/A4/A5 on chromosome 11q23 plays a pivotal role in TG metabolism (*5*), and apolipoprotein A5 (APO A5) has emerged as an important modulator of serum TG concentration (*6*). The APO A5 protein is predominantly synthesized in the liver. Overexpression of the APO A5 gene in mice resulted in elevated levels of plasma APO A5 and a marked decrease in plasma TG concentration. A 4-fold increase of serum TG levels can be found in the APO A5 knockout mice (*6,7*). In humans, variations of the

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APO A5 gene have been found to be associated with serum TG concentrations across ethnic groups (*8-10*).

APO A5 -1131T>C and S19W polymorphisms have been reported to be associated with an increased risk of CAD in multiple ethnic populations probably through its association with hypertriglyceridemia (*11-14*). However, there are also discrepant reports of no association between APO A5 -1131T>C and S19W polymorphisms and CAD risk (*15-17*). Therefore, the relation between APO A5 -1131T>C and S19W polymorphisms and risk of CAD remains controversial. To elucidate this discrepancy, we performed a meta-analysis of all available case-control studies to explore the association between the APO A5 polymorphisms and risk of CAD.

2. Materials and Methods

2.1. *Study Selection*

To identify all the articles that examined association of APO A5 polymorphisms with coronary artery disease, we conducted a comprehensive search of PubMed and EMBASE (the last searching update was May 28, 2011). Search terms included *apolipoprotein A-V or apolipoprotein AV or apolipoprotein A5 or APOAV or APOA-V or APO A5; gene, polymorphism, or genetic variant; and myocardial infarct, myocardial infarction, coronary artery disease, coronary heart disease, myocardial ischemia, ischemic heart disease, angina, acute coronary syndrome, acute coronary syndromes, ACS, coronary calcification, coronary flow reserve, ischemic heart failure, heart failure, or ischemic cardiomyopathy*. We also screened references of the retrieved articles and review articles by a hand search.

Eligible studies were included that fulfilled the following criteria: (i) association studies using an unrelated case-control design; (ii) complete data with genotype and allele frequencies. Cases were CAD, with the diagnosis based on angiographic or clinical criteria. Data from a study presented only in the form of an abstract and duplication studies were not included. Studies without genotype frequency were not included if the relevant information could not be obtained from the authors.

2.2. *Data extraction*

For each study, that met our criteria, the following information was collected: first author, year of publication, country of origin, ethnicity, criteria of diagnosis, number of cases and controls, genotype distribution, genotyping methods and allele frequency. All the searching work and data extraction work were conducted by two independent investigators (Zhang and Peng), and they reached a consensus on all items.

2.3. *Statistical analysis*

The strength of association between APO A5 polymorphisms and CAD was measured by odds ratio (OR) corresponding to a 95% confidence interval (CI) according to the method of Woolf (*18*). Heterogeneity between studies was assessed by Cochran's χ^2-based Q statistic test (*19*). Where *p*-value for heterogeneity was less than 0.05, a random-effects model using the DerSimonian and Laird method (*20*) was used to pool the results; otherwise, a fixed-effects model using the Mantel-Haenszel method was adopted (*21*). In order to better evaluate the extent of heterogeneity between studies, the I^2 test was also used. This statistic yields results ranged from 0 to 100% (I^2 = 0-25%, no heterogeneity; I^2 = 25-50%, moderate heterogeneity; I^2 = 50-75%, large heterogeneity; I^2 = 75-100%, extreme heterogeneity) (*22*). For the APO A5 -1131T>C promoter polymorphism, we investigated associations between the genetic variant and coronary artery disease risk in a recessive genetic model (C/C *vs.* C/T + T/T), dominant genetic model (C/C + C/T *vs.* T/T) and allelic contrast (C *vs.* T). For the APO A5 S19>W polymorphism, we investigated associations between the genetic variant and coronary artery disease risk in a recessive genetic model (W/W *vs.* W/S + S/S), dominant genetic model (W/W + W/S *vs.* S/S) and allelic contrast (W *vs.* S). The significance of the pooled OR was determined by the Z-test ($p < 0.05$ suggests a significant association).

Hardy-Weinberg equilibrium (HWE) was tested by χ^2 test at a significant level of $\alpha < 0.05$. Publication bias was investigated by funnel plots (*23*) and by Egger's linear regression test (*24*).

To examine specific subsets in these studies, separate analyses were used. A sensitivity analysis was performed to assess the influence of each study in which an individual study was removed each time. Likewise, a cumulative analysis was performed according to the ascending date of publication to identify influence of the first published study on subsequent publications and evolution of the combined estimates over time (*25*). Statistical analyses were all carried out using the STATA software package v 10.0 (Stata Corporation, College Station, TX). All *p*-values were two sided.

3. Results

3.1. *Study characteristics*

One hundred and thirty-three eligible studies were identified by our search strategy. One hundred and eight studies were excluded after title and abstract screening using the predefined inclusion and exclusion criteria. Then full text articles were retrieved for assessment in detail. In the end, 13 studies which investigated the association between the APO A5 -1131T>C

polymorphism and risk of CAD were selected in this meta-analysis with 5,050 cases and 7,272 controls (*11,12,14,16,17,26-33*). For the S19W APOA5 gene polymorphisms, 5 studies were included in the meta-analysis with 2,196 cases and 3,933 controls (*14,16, 17,27,34*). Characteristics of included studies are summarized in Table 1 and Table 2. Polymerase chain reaction-restriction fragment length polymorphism was the most commonly used genotyping method in these studies.

3.2. *Main Results, Sensitivity, and Cumulative Analyses*

For the APO A5 -1131T>C polymorphism and its relationship to CAD, significant heterogeneity was found under the recessive genetic model (I^2 = 52.2%, p = 0.014), and dominant genetic model (I^2 = 51.6%, p = 0.016). Therefore, the random-effects model (DerSimonian and Laird) was applied. No significant heterogeneity was found under allelic contrast (I^2 = 26.4%, p = 0.177) by the Mantel-Haenszel fixed effects model.

Significant statistical association was observed between the APO A5 -1131T>C polymorphism and CAD under the recessive genetic model (C/C *vs.* C/T + T/T, OR = 1.73, 95% CI = 1.37-2.19) (Figure 1). The same overall patterns were also observed under the dominant genetic model (C/C + C/T *vs.* T/T, OR = 1.42, 95% CI = 1.25-1.61) (Figure 2) and allelic contrast (C *vs.* T, OR = 1.31, 95% CI = 1.22-1.39) (data not shown).

After restricting our analysis to Chinese individuals, associations were found stronger under the recessive genetic model (C/C *vs.* C/T + T/T, OR = 1.84, 95% CI = 1.47-2.30) and allelic contrast (C *vs.* T, OR = 1.39, 95% CI = 1.21-1.60) (data not shown).

When stratified by status of HWE (the presence or absence of HWE in controls), no significant heterogeneity was found under the recessive genetic model (presence of HWE: I^2 = 26.5%, p = 0.192; absence of HWE: I^2 = 0%, p = 0.376) in both groups, and the positive association still existed (data not shown). The same patterns were also observed under the dominant genetic model (data not shown).

To investigate the influence of individual data of the APO A5 -1131T>C polymorphism sets on the pooled ORs, we deleted a single study involved in the meta-analysis each time. No individual study had an undue influence on the summary ORs under the recessive genetic model, dominant genetic model or allelic contrast (Table 3). We also performed a cumulative meta-analysis to identify the influence of the initial study on the subsequent publications. The influential role of the study of Bi *et al*. (*11*) was obvious in the cumulative random effects meta-analysis under the recessive genetic model, and the study of Hubacek *et al.* (*27*) was obvious under the dominant genetic model (Table 3).

For the APO A5 S19>W polymorphism and its relationship to CAD, significant heterogeneity was found under the dominant genetic model (I^2 = 76.3%, p = 0.002) and allelic contrast (I^2 = 75.7%, p = 0.002), but not found under the recessive genetic model (I^2 = 76.3%, p = 0.002). We observed no statistical association between APO A5 S19>W polymorphism and risk of CAD under the dominant genetic model (W/W + W/S *vs.* S/S, OR = 1.23, 95% CI = 0.76-2.00) and allelic contrast (W *vs.* S, OR = 1.30, 95% CI = 0.83-2.05), but strong association under the recessive genetic model (W/W *vs.* W/S + S/S, OR = 6.39, 95% CI = 2.68-15.24).

3.3. *Publication bias*

For the APO A5 -1131T>C polymorphism, the shape of funnel plots showed no obvious asymmetry and the result of the Egger's test did not show statistical evidence for bias either (Figure 3). For the APO A5 S19>W polymorphism, no publication bias was detected (data not shown).

4. Discussion

To the authors' knowledge, this is the first meta-analysis investigating the association between the APO A5 polymorphisms and CAD. In the present study, the effect of allele frequency and the effects of the dominant and recessive models were estimated. This meta-analysis reveals that the minor allele frequency of the -1131T>C polymorphism in the promoter of the APO A5 gene significantly increased the susceptibility for CAD. This effect was more pronounced in Chinese subjects.

The APO A5 -1131T>C polymorphism has been reported to be associated with the risk of CAD. This association has been shown to be mediated by TG levels in human studies. Bi *et al.* (*11*) found that CC homozygotes had approximately a twofold CAD risk compared with subjects with the TT genotype and the -1131C allele was correlated with increased levels of plasma TG in Chinese subjects. In a recent study, Jang *et al.* (*32*) reported that the homozygosity of the -1131C allele was associated with 47% higher TG as compared with TT subjects in Korean CAD patients. Similarly, our findings suggest that there is a modest association between the APO A5 -1131T>C polymorphism and CAD (recessive genetic model: OR = 1.73, 95% CI = 1.37-2.19; dominant genetic model: OR = 1.42, 95% CI = 1.25-1.61; allelic contrast: OR = 1.31, 95% CI = 1.22-1.39, respectively). In a recent meta-analysis of the association of the APO A5 -1131T>C polymorphism and fasting blood lipids, Zhao *et al*. found a strong association of the APO A5 -1131 T>C polymorphism with higher levels of TG (*35*). The -1131T>C polymorphism is located in the promoter

Table 1. Characteristics of included studies of APO A5 -1131T>C polymorphism

Author	Year	Population	Average age		Gender component*	Number of sample			Genotypes for Cases, *n*			Genotypes for Controls, *n*			Frequency of C Allele		HWE in Control	
			Case	Control		Case	Control	Total	TT	TC	CC	TT	TC	CC	Case	Control	χ^2	*p*
Bi *et al.* (*11*)	2004	Chinese	60.2	58.9	209/103/191/126	312	317	629	108	159	45	136	151	30	0.399	0.333	1.671	0.196
Hubacek *et al.* (*27*)	2004	Caucasians	55.1	49.0	435/0/1,191/1368	435	2,559	2,994	366	46	23	2,164	355	40	0.106	0.085	29.905	0.000
Szalai *et al.* (*12*)	2004	Hungarian	57.5	58.5	236/72/235/75	308	310	618	248	53	7	277	31	2	0.109	0.056	1.165	0.281
Liu *et al.* (*14*)	2005	Chinese	54.2	54.4	285/198/276/226	483	502	985	181	226	76	246	212	44	0.391	0.299	0.031	0.861
Tang *et al.* (*28*)	2005	Chinese	63.6	60.9	158/77/163/99	235	262	497	80	120	35	107	130	25	0.404	0.344	2.627	0.105
Yan *et al.* (*29*)	2005	Chinese	65.0	52.0	46/67/70/85	113	155	268	41	60	12	83	58	14	0.372	0.277	0.689	0.407
Hsu *et al.* (*26*)	2006	Chinese	61.6	61.0	161/50/242/75	211	317	528	104	83	24	145	156	16	0.310	0.297	10.221	0.001
Yu *et al.* (*30*)	2007	Chinese	52.1	51.5	94/46/97/59	140	156	296	46	67	27	67	75	14	0.432	0.330	1.181	0.277
Martinelli *et al.* (*17*)	2007	Italian	60.7	58.7	544/125/168/76	669	244	913	545	118	6	204	37	3	0.097	0.088	0.776	0.378
Jang *et al.* (*32*)	2009	Korean	55.2	55.2	665/76/6,65/76	741	741	1,482	320	343	78	382	295	64	0.337	0.285	0.428	0.513
Ashokkumar *et al.* (*31*)	2009	Indian	53.2	53.6	322/94/315/101	416	416	832	191	183	42	239	155	22	0.321	0.239	0.235	0.628
Prochaska *et al.* (*16*)	2010	Brazilian	60.1	58.3	112/68/95/75	180	170	350	150	27	3	147	22	1	0.092	0.071	0.032	0.858
Park *et al.* (*33*)	2010	Korean	56.9	56.1	658/149/880/243	807	1,123	1,930	363	367	77	566	455	102	0.323	0.293	0.587	0.444

*Gender component: number of male cases/number of female cases/number of male controls/number of female controls.

Table 2. Characteristics of included studies of APO A5 S19>W polymorphism

Author	Year	Population	Average age		Gender component*	Number of sample			Genotypes for Cases, *n*			Genotypes for Controls, *n*			Frequency of W Allele		HWE in Control	
			Case	Control		Case	Control	Total	SS	SW	WW	SS	SW	WW	Case	Control	χ^2	*p*
Hubacek *et al.* (*27*)	2004	Caucasians	55.1	49	435/0/1191/1,368	435	2,559	2,994	369	56	10	2,198	352	9	0.087	0.072	1.66	0.197
Liu *et al.* (*14*)	2005	Chinese	54.2	54.4	285/198/276/226	483	502	985	439	43	1	502	0	0	0.047	0	-	-
Dallongeville *et al.* (*34*)	2006	French	-	-	429/0/458/0	429	458	887	368	56	5	414	44	0	0.077	0.048	1.17	0.28
Martinelli *et al.* (*17*)	2007	Italian	60.7	58.7	544/125/168/76	669	244	913	605	59	5	219	25	0	0.052	0.051	0.71	0.399
Prochaska *et al.* (*16*)	2010	Brazilian	60.1	58.3	112/68/95/75	180	170	350	157	23	0	145	25	0	0.064	0.074	1.07	0.301

*Gender component: number of male cases/number of female cases/number of male controls/number of female controls.

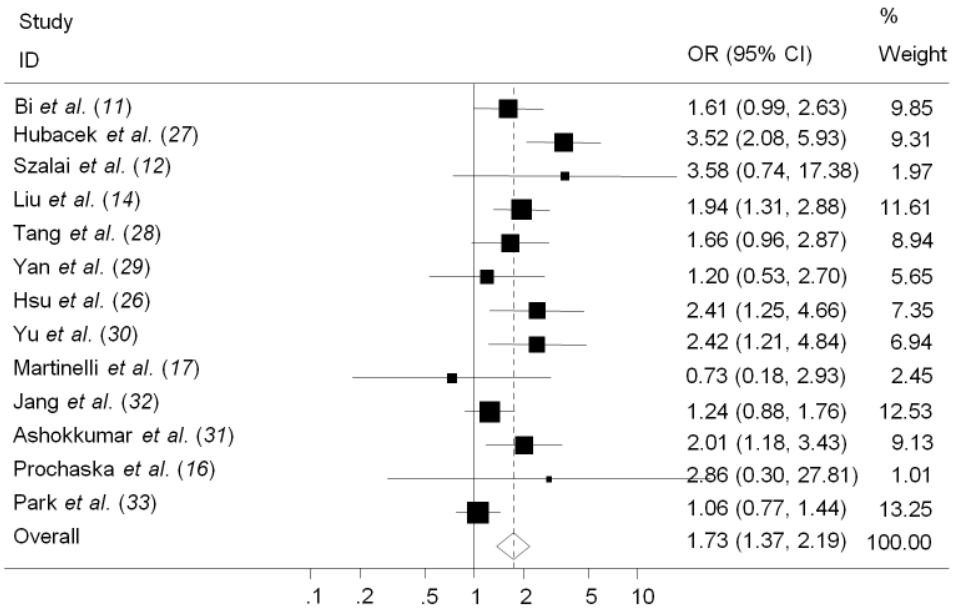

Figure 1. Random effects odds ratio (OR) for association between the APO A5 -1131T>C polymorphism and risk of CAD (C/C *vs.* C/T + T/T). Size of the gray box is proportional to weight of the corresponding study. Pooled estimate is displayed as a diamond. Bars, 95% confidence interval (CI).

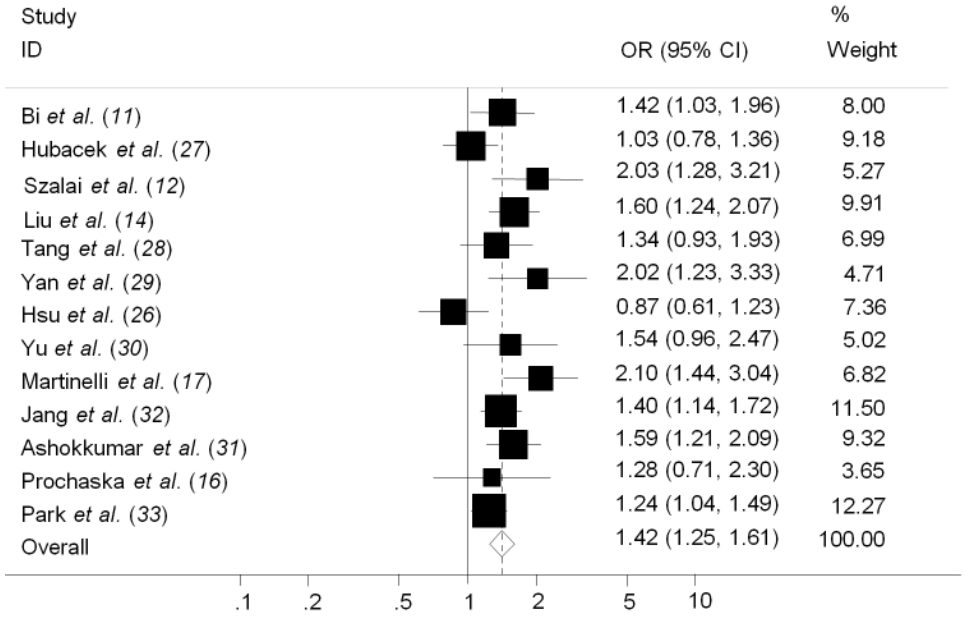

Figure 2. Random effects odds ratio (OR) for association between the APO A5 -1131T>C polymorphism and risk of CAD (C/C + C/T vs T/T). Size of the gray box is proportional to weight of the corresponding study. Pooled estimate is displayed as a diamond. Bars, 95% CI.

region of the APO A5 gene and there is no transcription factor binding sites identified in this location. Therefore, it is justified that the -1131T>C variant may not be functional. However, -1131C may affect the transcriptional activity of the APO A5 gene. In addition, Vaessen *et al.* (*10*) has suggested that association of -1131T>C with CAD is likely attributable to linkage disequilibrium with APOC3 variants or to other closely linked genetic variations.

In the present study, the minor allele frequency of the APO A5 -1131T>C polymorphism has been found to be a stronger association with the risk of CAD in Chinese subjects. This may due to higher frequency of the C allele in Chinese as compared with others (case: 0.372-0.432; control: 0.277-0.344, respectively). As the CAD mortality rates in China have dramatically increased in recent years (*36*), the presence of the APO A5 -1131C allele may have more impact on CAD risk

Table 3. Sensitivity and cumulative analyses for contrast of different genetic models of APO A5 -1131T>C polymorphism

Authors	Sensitivity analysis			Cumulative analysis		
	C/C *vs.* C/T + T/T (recessive genetic model)	C/C + C/T *vs.* T/T (dominant genetic model)	C *vs.* T (allelic contrast)	C/C *vs.* C/T + T/T (recessive genetic model)	C/C + C/T *vs.* T/T (dominant genetic model)	C *vs.* T (allelic contrast)
Bi *et al.* (*11*)	1.60 (1.37, 1.86)	1.38 (1.26, 1.50)	1.30 (1.22, 1.39)	1.61 (0.98, 2.63)	1.41 (1.02, 1.95)	1.33 (1.05, 1.67)
Hubacek *et al.* (*27*)	1.50 (1.28, 1.74)	1.42 (1.30, 1.55)	1.31 (1.22, 1.40)	2.32 (1.62, 3.32)	1.18 (0.95, 1.46)	1.31 (1.11, 1.54)
Szalai *et al.* (*12*)	1.59 (1.37, 1.84)	1.36 (1.25, 1.48)	1.29 (1.21, 1.38)	2.37 (1.67, 3.36)	1.30 (1.07, 1.57)	1.38 (1.19, 1.62)
Liu *et al.* (*14*)	1.55 (1.32, 1.82)	1.36 (1.24, 1.48)	1.28 (1.19, 1.37)	2.17 (1.67, 2.82)	1.40 (1.20, 1.63)	1.43 (1.27, 1.61)
Tang *et al.* (*28*)	1.59 (1.37, 1.86)	1.38 (1.27, 1.51)	1.30 (1.22, 1.39)	2.06 (1.63, 2.61)	1.39 (1.21, 1.60)	1.41 (1.26, 1.57)
Yan *et al.* (*29*)	1.61 (1.39, 1.87)	1.37 (1.25, 1.49)	1.30 (1.21, 1.38)	1.98 (1.57, 2.48)	1.43 (1.25, 1.64)	1.42 (1.28, 1.57)
Hsu *et al.* (*26*)	1.56 (1.35, 1.82)	1.42 (1.30, 1.55)	1.32 (1.24, 1.41)	2.02 (1.63, 2.50)	1.34 (1.18, 1.52)	1.36 (1.24, 1.50)
Yu *et al.* (*30*)	1.57 (1.35, 1.82)	1.38 (1.26, 1.50)	1.30 (1.21, 1.38)	2.05 (1.67, 2.52)	1.35 (1.19, 1.52)	1.38 (1.25, 1.51)
Martinelli *et al.* (*17*)	1.61 (1.39, 1.87)	1.35 (1.24, 1.47)	1.31 (1.23, 1.40)	2.01 (1.64, 2.46)	1.41 (1.25, 1.58)	1.36 (1.24, 1.49)
Jang *et al.* (*32*)	1.69 (1.44, 1.98)	1.38 (1.26, 1.51)	1.31 (1.22, 1.41)	1.78 (1.49, 2.12)	1.40 (1.27, 1.55)	1.33 (1.23, 1.44)
Ashokkumar *et al.* (*31*)	1.57 (1.35, 1.83)	1.36 (1.25, 1.49)	1.29 (1.20, 1.38)	1.80 (1.52, 2.12)	1.43 (1.30, 1.57)	1.35 (1.26, 1.46)
Prochaska *et al.* (*16*)	1.59 (1.38, 1.85)	1.38 (1.27, 1.50)	1.30 (1.22, 1.39)	1.80 (1.53, 2.13)	1.42 (1.29, 1.56)	1.35 (1.26, 1.45)
Park *et al.* (*33*)	1.80 (1.52, 2.13)	1.42 (1.29, 1.56)	1.35 (1.26, 1.45)	1.60 (1.38, 1.85)	1.38 (1.27, 1.50)	1.30 (1.22, 1.39)

*Gender component: number of male cases/number of female cases/number of male controls/number of female controls.

Figure 3. Begg's funnel plot for publication bias test of the APO A5 -1131T>C polymorphism with pseudo 95% confidence limits. (A) recessive genetic model. **(B)** dominant genetic model. Each point represents a separate study for the indicated association. Horizontal line represents the mean effects size.

in Chinese subjects. When stratified by status of HWE, the positive association still existed, but significant heterogeneity losses were found in both groups, suggesting that the status of HWE might be a potential source of between-study heterogeneity.

The present meta-analysis also supported an association between the APO A5 S19>W polymorphism and its relationship to CAD: the minor allele under a recessive model provided evidence of risk. This result should be interpreted with some degree of caution, because the numbers of studies and participants were relatively small.

The present study has some limitations. First, though we have collected all eligible studies, the number of qualified studies was not large. Second, all included studies had a case-control design. Third, although we had designed our study to evaluate effects of environmental modification such as smoking, alcohol intake, physical activities, and diets, few investigators reported the effects of these environmental factors and the definition of each stratum varied too much among studies. We failed to analyze modification of the effects of this polymorphism by environment factors.

In spite of the limitations, our meta-analysis has some key advantages. First, the results should be more reliable than those from a single study, as cases and controls were pooled from different studies and statistical power of analysis was significantly increased. Second, no publication bias was found. Sensitive analyses conducted by deselecting studies one by one in chronological order found no significant changes and reversal of results, which suggested the result of the present meta-analysis, was stable and reliable.

In conclusion, this meta-analysis suggests that the minor allele of the APO A5 -1131T>C polymorphism is a risk factor for CAD, especially in the Chinese population. Primary studies of a large population are required to further evaluate gene-gene and gene-environment interactions of this polymorphism on CAD risk in different ethnicities.

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Original Article

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Valsartan attenuated oxidative stress, decreased MCP-1 and TGF-β_1 expression in glomerular mesangial and epithelial cells induced by high-glucose levels

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Summary **Our previous studies revealed that valsartan, an angiotensin II type I receptor blocker, exhibited renoprotective effects through decreasing urine protein excretion levels due to improving glomerular permeability in rats with diabetic nephropathy (DN). In this study, we sought to investigate the underlying mechanisms in perspectives of oxidative stress, transforming growth factor beta-1 (TGF-β_1) and monocyte chemoattractant protein-1 (MCP-1) expressions in glomerular mesangial cells (GMCs) and glomerular epithelial cells (GECs) since their roles are well-established in the development and progression of DN. High-glucose levels significantly increased oxidative stress in GMCs and GECs, as evidenced by enhanced generation of reactive reactive oxygen species (ROS), reduced levels of glutathione (GSH) and antioxidant enzyme superoxide dismutase (SOD), and increased production of malondialdehyde (MDA). Treatment with valsartan significantly restored the levels of those oxidative stress relevant molecules. Furthermore, valsartan obviously diminished the expression of proinflammatory cytokine MCP-1 in GMCs and GECs induced by high-glucose levels both at mRNA and protein levels, as determined by real-time PCR, immunocytochemistry, western blotting, and ELISA. In addition, the increased expressions of TGF-β_1 mRNA and protein induced by high-glucose level were also abrogated by valsartan treatment in GMCs, as evaluated by real-time PCR and ELISA. These results suggest that the renoprotective effects of valsartan may be related to its potential in decreasing oxidative stress and the expressions of MCP-1 and TGF-β_1 in GMCs and GECs.**

Keywords: Diabetic nephropathy, valsartan, glomerular mesangial cells, glomerular epithelial cells, oxidative stress, MCP-1, TGF-β_1

1. Introduction

Diabetic nephropathy (DN) is the most common complication of diabetes mellitus, often leading to end-stage kidney disease and a high risk of mortality (*1,2*). It is characterized clinically by progressively increasing albuminuria and histopathologically by glomerular basement membrane (GBM) thickening and mesangial expansion due to accumulation of extracellular matrix (ECM) proteins (*3*). Functional changes in diabetic glomeruli, particularly in glomerular mesangial cells (GMCs) and glomerular epithelial cells (GECs) were demonstrated to exert critical roles in the development and progression of DN (*4,5*). On the one side, an enhancement of the production of ECM has been shown in GMCs under high-glucose conditions (*6*). On the other side, the damage of GECs which function as a fine filter contributing ultimate size-selectivity, permitting permeability to molecules smaller than albumin in the normal physiological state, leads to retraction of their foot processes and proteinuria (*7*). Thus, GMCs and GECs have been the focus in the field of research on DN.

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Previous studies indicated that transforming growth factor beta (TGF-β), oxidative stress, and proinflammatory cytokines, such as monocyte chemoattractant protein-1 (MCP-1), play important roles in progressive DN (*8-11*). The knowledge and control of these different mechanisms have become a fascinating therapeutic challenge, aimed to reduce the progression of DN. Although no curable therapy is yet available, an increasing number of reports indicate that blockade of the renin angiotensin system (RAS) is effective to delay the progression of DN and thereby to protect against end-stage renal failure. Treatment with angiotensin II (Ang II) type I receptor (AT1R) blockers (ARB), *e.g.* valsartan, by the current authors and other researchers, has been shown to have protective effects against the progression of DN through improving glomerular permeability and thus decreasing urine protein excretion levels (*12,13*). However, besides their well-documented efficiency, the underlying mechanisms remain to be elucidated.

In the present study, we first studied the effects of valsartan against oxidative stress in GMCs and GECs cultured in high-glucose conditions by evaluating levels of reactive oxygen species (ROS), glutathione (GSH), antioxidant enzyme superoxide dismutase (SOD), and malondialdehyde (MDA). Next, the expression of proinflammatory cytokine MCP-1 was measured in GMCs and GECs treated with or without valsartan. Additionally, the change of expression levels of TGF-β_1 was determined in GMCs after treatment with valsartan.

2. Materials and Methods

2.1. *Chemicals*

Valsartan was purchased from Changzhou Kony Pharm Co., Ltd. (Changzhou, Jiangsu, China). Valsartan was dissolved in dimethyl sulfoxide (DMSO) before use. The final concentration of DMSO in the cell culture media is ≤ 3‰ (v/v).

2.2. *Cell culture*

Rat GMCs (HBZY-1) were purchased from Chinese Center for Typical Culture Collection (Wuhan, Hubei, China). GMCs were maintained in normal-glucose (5.6 mmol/L D-glucose) RPMI-1640 media supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) at 37°C in a humid atmosphere (5% CO_2-95% air). Conditionally immortalized mouse GECs were kindly provided by Dr. Peter Mundel, Department of Medicine, Mount Sinai School of Medicine, New York, USA. Cultivation of GECs was performed as described previously (*14*). To induce proliferation, cells were cultured on type I collagen-coated dishes in normal-glucose RPMI-1640 medium supplemented with 10% FCS and 10 U/mL murine interferon-γ (IFN-γ) at 33°C in a humid atmosphere (5% CO_2-95% air). To induce differentiation, cells were maintained in normal-glucose RPMI-1640 with 10% FCS but without IFN-γ at 37°C for a period of two weeks without cell passage. Cells were identified as differentiated GECs by their arborized morphology and the presence of high levels of synaptopodin determined by an immunofluorescence assay (data not shown). To mimic the diabetic state, GMCs and differentiated GECs were pretreated in high-glucose (30 mmol/L D-glucose) RPMI-1640 medium with 10% FCS before the experiments.

For experiments, GMCs and GECs were distributed into three groups, respectively, and each group included two parallel samples: *i*) Normal group: cells were cultured in normal-glucose medium during the entire study; *ii*) Model group: cells pretreated with high-glucose medium were cultured in high-glucose medium without valsartan; *iii*) Valsartan group: cells pretreated with high-glucose medium were cultured in high-glucose medium with addition of 10^{-6} mol/L valsartan. Each experiment was repeated four times.

2.3. *Determination of ROS*

ROS production was assessed using the fluorescent probe 6-carboxy-2,7-dichlorodihydrofluorescein diacetate (CDCFH-DA) (Molecular Probes, Eugene, OR) (*15*). GMCs and GECs (1×10^4 per well) were seeded in 24-well plates, respectively. After cells were allowed to attach, the specified concentration of valsartan was added to the wells and incubated for 48 h. Cells were then rinsed twice with phosphate-buffered saline (PBS) and replaced with phenol red free RPMI-1640 containing 20 mM CDCFH-DA. After 60 min incubation, the fluorescence intensity was measured with a fluorescence microplate reader CytoFluor 2350 (Millipore, Bedford, MA, USA) with excitation and emission wavelengths of 502 and 530 nM, respectively.

2.4. *Determination of SOD, GSH, and MDA levels*

GMCs and GECs (1×10^4 per well) were seeded in 24-well plates, respectively. After cells were allowed to attach, the specified concentration of valsartan was added to the wells and incubated for 48 h. Then the cell culture supernatant was collected for determination of SOD, GSH, and MDA levels.

Total GSH content was determined spectrophotometrically using the method described by Akerboom and Sies (*16*). The assay mixture in 1 mL contained 200 μL cell culture supernatant, 730 μL 0.1 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 50 μL 0.5% $NaHCO_3$ containing 4 mg/mL NADPH, and 20 μL 0.5% $NaHCO_3$ containing 1.5 mg/mL 5,5-dithiobis (2-nitrobenzoicacid) (DTNB) (Sigma-Aldrich, USA). The reaction was started by adding 6

units of GSH reductase and lasted for 1 min at 25°C. Then the absorbance was measured at 412 nM using a Multilabel Plate Counter VICTOR31420 (Perkin-Elmer, Waltham, Massachusetts, USA).

SOD activity was determined using a commercially available SOD kit (RANSOD SD125, Randox, Antrim, UK). The method is based on the formation of red formazan from the reaction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride and superoxide radical (produced in the incubation medium from the xanthine-xanthine oxidase reaction system), which is assayed spectrophotometrically at 505 nM using a Multilabel Plate Counter VICTOR31420 (Perkin-Elmer, Waltham, Massachusetts, USA). The inhibition of the produced chromogen is proportional to the activity of SOD present in the sample. Analysis was performed according to the manufacturer's recommended protocol.

The quantification of lipid peroxidation was estimated by determining malondialdehyde (MDA) reacting to thiobarbituric acid (TBA)-reactive substance following the method described by Ohkawa (*17*). Briefly, an aliquot of 200 μL of cell culture supernatant was mixed thoroughly with an aqueous solution of TBA and heated at 95°C for 30 min in a water bath. The suspension was then cooled to room temperature, centrifuged at 4,000 r/min for 10 min, and the pink colored supernatant was measured spectrophotometry at 532 nM. Absorbance was determined using a Multilabel Plate Counter VICTOR31420 (Perkin-Elmer, Waltham, Massachusetts, USA). MDA concentration was calculated using the absorbance coefficient of MDA-TBA complex (absorbance coefficient = $1.56 \times 105\ M^{-1}\ cm^{-1}$).

2.5. *Real-time PCR*

GMCs and GECs (5×10^5 per well) were seeded in 6-well plates, respectively. After cells were allowed to attach, the specified concentration of valsartan was added in the wells and incubated for 48 h. Cells were then collected and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Two μg of total RNA was reverse-transcribed into cDNA by Super Script III first strand cDNA synthesis Kit (Invitrogen, Carlsbad, CA, USA). Real-time PCR was performed with the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The SYBR Green PCR Master Mix kits (Applied Biosystems, Foster City, CA, USA) were used according to the supplier's instructions for quantification of gene expression. Primer pairs used are as follows: for MCP-1, F: 5-GATCTCAGTGCAGAGGCTCG-3 and R: 5-TGCTTGTCCAGGTGGTCCAT-3; for TGF-β_1, F: 5-TGGCGTTACCTTGGTAACC-3 and R: 5-GGTGTTGAGCCCTTTCCAG-3; for β-actin, F: 5-GGCTGTATTCCCCTCCATCG-3 and R: 5-CCAGTTGGTAACAATGCCATGT-3; for GADPH, F: 5-TCCCTCAAGATTGTCAGCAA-3 and R: 5-AGATCCACAACGGATACATT-3. Thermal cycling conditions were as follows: 95°C for 2 min; 40 cycles of 10 s denaturation at 94°C, 10 s annealing at 54°C, and 20 s extension at 72°C; and 1 cycle of 5 min at 72°C. The calculation of the relative expression level of MCP-1 was conducted based on the cycle threshold (C_t) method. The relative mRNA levels of MCP-1 and TGF-β_1 were expressed as ratios compared with GAPDH and β-actin mRNA levels, respectively.

2.6. *Immunocytochemistry*

GMCs (5×10^5 per well) were seeded in 6-well plates in which sterilized coverslips were pro-positioned. After cells were allowed to attach, the specified concentration of valsartan was added to the wells and incubated for 48 h. Coverslips were then taken out and washed twice with PBS. GMCs were fixed with 4% paraformaldehyde for 1 h at 4°C. After incubation with anti-MCP-1 (Boster, Wuhan, China) at 4°C, the cells were washed and treated with biotinylated anti-immunoglobulin, washed, reacted with avidin-conjugated horseradish peroxidase H complex, and incubated in diaminobenzidine and hydrogen peroxide. Cells were then rinsed in distilled water and counterstained with hematoxylin. Images were captured and the average grey scale was quantified by means of a computer-assisted image analyzer, Image Pro Plus 5.1 (Media Cybernetics, Inc., Bethesda, MD, USA).

2.7. *ELISA*

Cells (5×10^5 per well) pretreated in high-glucose medium were seeded in 6-well plates. After cells were allowed to attach, the specified concentration of valsartan was added to the wells and incubated for 48 h. MCP-1 protein level in the culture supernatant was determined using commercially available ELISA MCP-1 and TGF-β_1 kits (Bionewtrans Pharmaceutical Biotechnology Co., Ltd., USA) according to the manufacturer's protocols. MCP-1 and TGF-β_1 protein levels were determined by comparing the samples to the standard curve generated by the kit.

2.8. *Western blot*

Western blotting was used to evaluate the expressions of MCP-1 in GECs. GECs (5×10^5 per well) were seeded in 6-well plates. After cells were allowed to attach, the specified concentration of valsartan was added to the wells and incubated for 48 h. The cells were harvested and cell lysates (30 μg of protein per lane) were fractionated using 10% SDS-PAGE. Proteins were electro-transferred onto nitrocellulose

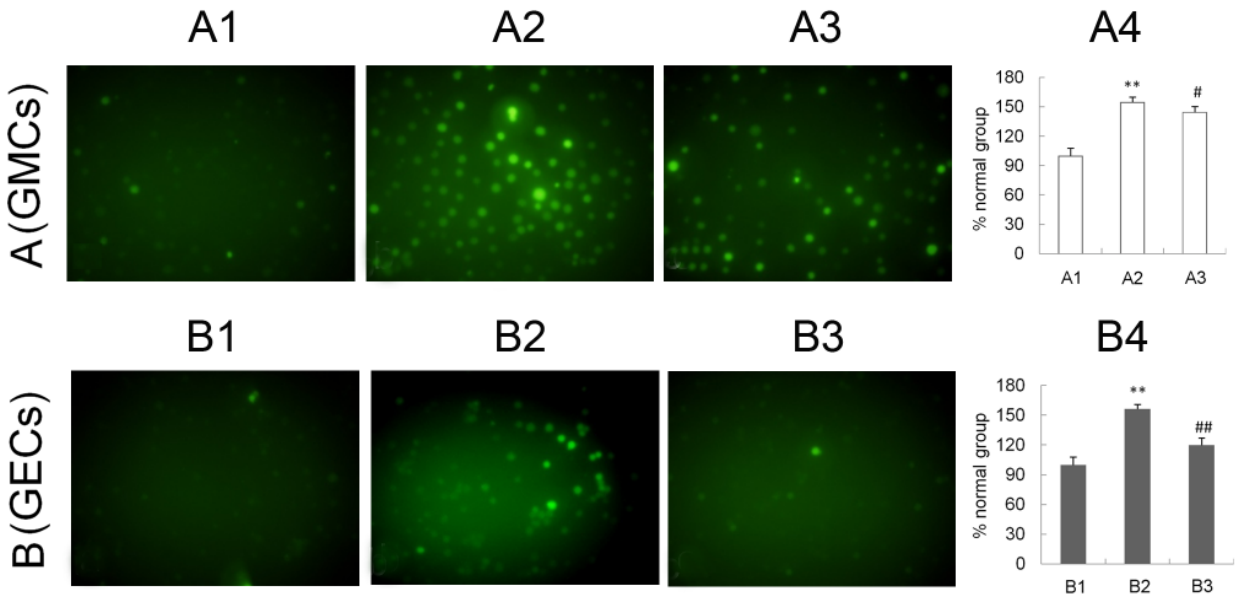

Figure 1. Effects of high-glucose levels and valsartan treatment on ROS production in GMCs (A) and GECs (B). A1 and **B1**, normal group; **A2** and **B2**, model group; **A3** and **B3**, valsartan group; **A4** and **B4**, quantification of fluorescence intensity in model and valsartan groups by comparing with that in normal group. $^{\#}$, $p < 0.05$; $^{\#\#}$, $p < 0.01$; **, $p < 0.01$.

membranes and then the level of MCP-1 expression was detected using a rabbit polyclonal antibody to MCP-1 (Santa Cruz, USA). Blots were washed in 0.05% Tween-20/PBS and then incubated with horseradish peroxidase-conjugated secondary antibody. β-actin protein level served as a protein loading control. The bound antibodies were visualized using an enhanced chemiluminescence reagent (Amersham Pharmacia Biotech, USA) followed by exposure to X-ray film. The band densities were measured using TINA image software (Raytest, Straubenhardt, Germany).

2.9. *Statistical analysis*

Data are expressed as mean ± S.D. One-way ANOVA followed by Dunnett's test was performed using SPSS/Win11.0 software (SPSS, Inc., Chicago, Illinois, USA); $p < 0.05$ was indicative of a significant difference.

3. Results

3.1. *Valsartan decreased oxidative stress induced by high-glucose level*

3.1.1. *Valsartan decreased high-glucose level induced ROS production*

Levels of ROS produced in GMCs and GECs were estimated using the fluorescent probe CDCFH-DA. Exposure of cultured GMCs to high-glucose conditions induced a significant increase in fluorescence intensity compared with exposure to normal-glucose conditions ($p < 0.01$, Figure 1A), suggesting a stimulatory effect of high glucose levels on free radical production. The fluorescence signal produced by GMCs cultured in high-glucose conditions was 1.60-fold of that produced by cells in normal-glucose conditions. After incubation of GMCs with valsartan at a concentration of 10^{-6} mol/L for 48 h, the fluorescence intensity was significantly decreased in GMCs (1.40-fold of that in normal group; $p < 0.05$), indicating that the ROS levels were significantly reduced.

Similar results were obtained in cultured GECs (Figure 1B). However, effects of valsartan in deceasing ROS levels in GECs were more potent than that in GMCs at the same concentration. An extremely significant difference in fluorescence intensity was showed between the model group (1.65-fold of that in the normal group) and the valsartan group (1.20-fold of that in normal group) ($p < 0.01$).

3.1.2. *Valsartan increased high-glucose level induced GSH reduction*

GSH content in cultured supernatant of GMCs (Figure 2A) and GECs (Figure 2B) was measured by the enzymatic recycling method in which GSH was oxidized by DTNB and reduced by NADPH. In normal-glucose conditions, GSH concentrations in GMCs and GECs were determined at 1.67 and 1.07 nmol/L, respectively. They were significantly decreased to 0.82 and 0.42 nmol/L in GMCs and GECs cultured in high-glucose conditions, respectively ($p < 0.01$). After incubation of GMCs and GECs with valsartan at a concentration of 10^{-6} mol/L for 48h, GSH content was obviously increased to 1.12 nmol/L and 0.61nmol/L ($p < 0.01$ in GMCs, $p < 0.05$ in GECs).

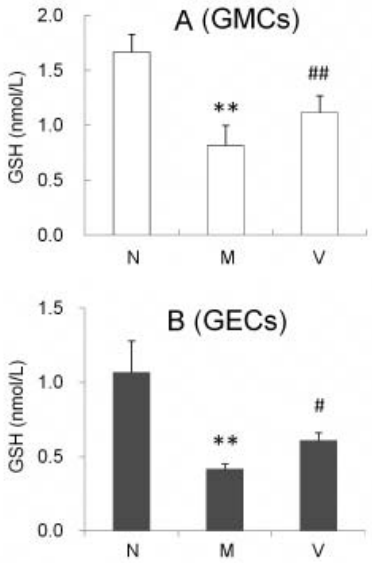

Figure 2. Effects of high-glucose levels and valsartan treatment on GSH content in GMCs (A) and GECs (B). Abbreviations: N, normal group; M, model group; V, valsartan group. #, $p < 0.05$; ##, $p < 0.01$; **, $p < 0.01$.

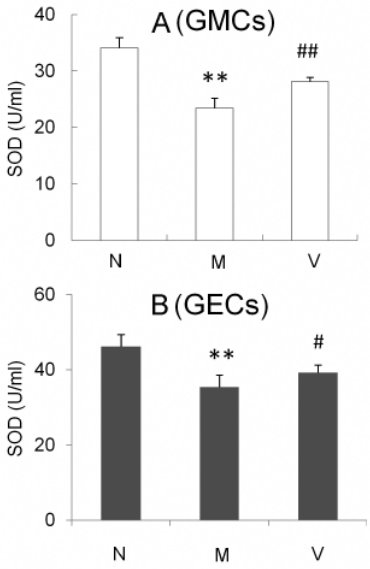

Figure 3. Effects of high-glucose levels and valsartan treatment on SOD activities in GMCs (A) and GECs (B). Abbreviations: N, normal group; M, model group; V, valsartan group. #, $p < 0.05$; ##, $p < 0.01$; **, $p < 0.01$.

3.1.3. *Valsartan increased high-glucose level induced lessened SOD activity*

SOD activity was evaluated by measuring the production of red formazan from the reaction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride and superoxide radical generated with the xanthine-xanthine oxidase reaction system. In normal-glucose conditions, SOD activities in GMCs (Figure 3A) and GECs (Figure 3B) were determined at 34.1 and 46.2 U/mL, respectively. SOD activities were significantly decreased to 23.5 and 35.4 U/mL in GMCs and GECs cultured in high-glucose conditions, respectively ($p < 0.01$). Incubation of GMCs and GECs with valsartan at a concentration of 10^{-6} mol/L for 48 h, SOD activities were obviously increased to 28.2 and 39.3 U/mL, respectively ($p < 0.01$ in GMCs, $p < 0.05$ in GECs).

3.1.4. *Valsartan improved high-glucose level induced oxidative damage*

MDA is produced by the hydrolysis of lipid hydroperoxides, which reacts with TBA to produce a complex that absorbs at 532 nM. In normal-glucose conditions, MDA levels in GMCs (Figure 4A) and GECs (Figure 4B) were determined at 2.32 and 2.10 mmol/L, respectively. They were significantly increased to 5.48 and 4.88 mmol/L in GMCs and GECs cultured in high-glucose conditions, respectively ($p < 0.01$). After incubation of GMCs and GECs with valsartan at a concentration of 10^{-6} mol/L for 48 h, MDA content was obviously decreased to 3.82 and 3.02 mmol/L, respectively ($p < 0.01$).

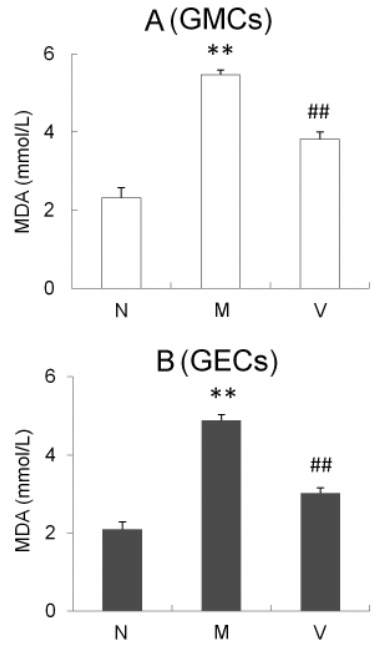

Figure 4. Effects of high-glucose levels and valsartan treatment on MDA production in GMCs (A) and GECs (B). Abbreviations: N, normal group; M, model group; V, valsartan group. ##, $p < 0.01$; **, $p < 0.01$.

3.2. *Valsartan diminished high-glucose induced MCP-1 expression*

Since MCP-1 is a well-known proinflammatory cytokine and its expression was reported to be increased under diabetic conditions (*11*), we attempted to elucidate whether valsartan is able to decrease high-glucose levels induced by MCP-1 expression in GMCs and GECs both at the protein and mRNA levels.

MCP-1 protein expressed by GMCs was evaluated using an immunocytochemistry method (Figure 5A). Exposure of cultured GMCs to high-glucose levels induced a significant increase of MCP-1 signal compared with exposure to normal-glucose levels ($p < 0.01$; Figure 5B). Incubation of valsartan for 48 h with GMCs cultured in high-glucose condition resulted in a significant decrease of MCP-1 signal ($p < 0.05$; Figure 5B). The mRNA levels of MCP-1 in GMCs were determined using a real-time PCR method. As demonstrated in Figure 5C, the ratio of mRNA levels of MCP-1 and GAPDH was determined to be 0.47 in normal-glucose cultured cells and this value was dramatically augmented to 1.63 in high-glucose cultured cells ($p < 0.01$). Valsartan significantly diminished MCP-1 mRNA expression induced by high-glucose levels, with a ratio of 1.14 ($p < 0.01$).

In GECs, MCP-1 mRNA and protein expressions were evaluated using real-time PCR, ELISA and Western blotting methods. As shown in Figure 6A, the ratio of mRNA levels of MCP-1 and GAPDH was increased from 0.91 in the normal group to 4.85 in the model group ($p < 0.01$). This value was remarkably decreased to 3.52 by addition of valsartan ($p < 0.05$). In accordance with mRNA expression, similar profiles of extracellular (Figure 6B) and intracellular (Figures 6C and 6D) MCP-1 protein expression were found in GECs. The extracellular MCP-1 protein concentration was significantly increased from 220 ng/μL in the normal group to 484 ng/μL in the model group ($p < 0.01$). MCP-1 concentration was obviously decreased in the valsartan treatment group with a value 385 ng/μL ($p < 0.05$). The intracellular MCP-1 protein level was demonstrated to be 2.5-fold in cells cultured in high-glucose conditions compared to that in cells cultured in normal-glucose conditions. Valsartan also dramatically reduced MCP-1 expression in GECs induced by high-glucose levels (1.6-fold of that in normal group) ($p < 0.05$).

3.3. *Valsartan diminished high glucose-induced TGF-β_1 expression*

TGF-β_1 mRNA and protein expression in GMCs were determined using real-time PCR and ELISA methods. As shown in Figure 7A, high-glucose levels induced a significant increase in the ratio of mRNA levels of TGF-β_1 and β-actin, from 0.38 to 0.92 compared with the normal-glucose level ($p < 0.01$). This ratio was significantly decreased to 0.80 after 48 h valsartan treatment ($p < 0.05$). In accordance with the mRNA level, the extracellular TGF-β_1 protein concentration was significantly increased from 54.9 pg/mL in the normal group compared to 88.2 pg/mL in the model group ($p < 0.01$), and decreased to 71.8 pg/mL due to valsartan treatment ($p < 0.05$) (Figure 7B).

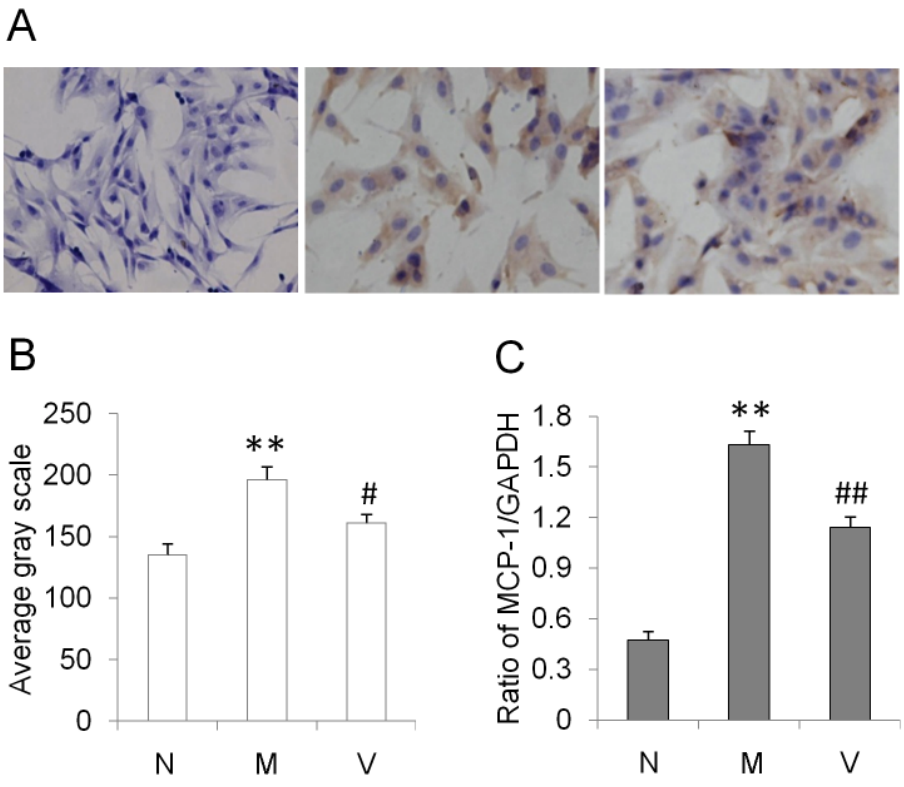

Figure 5. Effects of high-glucose levels and valsartan treatment on MCP-1 protein (A, B) and mRNA (C) expressions in GMCs. Abbreviations: N, normal group; M, model group; V, valsartan group. $^{\#}$, $p < 0.05$; $^{\#\#}$, $p < 0.01$; **, $p < 0.01$.

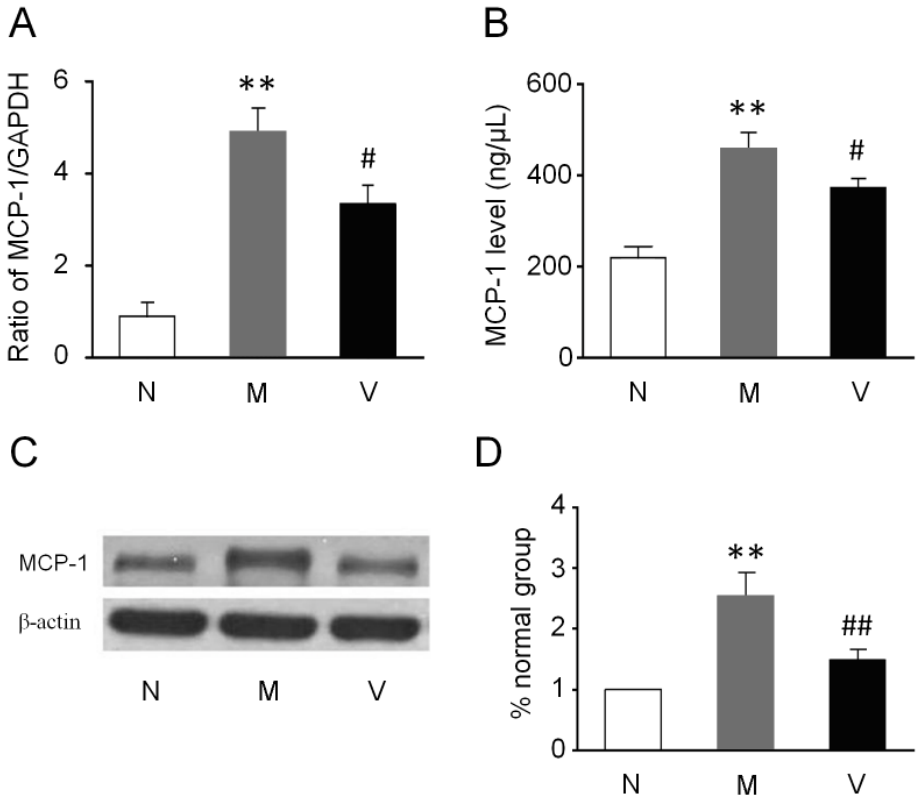

Figure 6. Effects of high-glucose levels and valsartan treatment on MCP-1 mRNA (A), extracellular (B) and intracellular (C, D) protein levels in GECs. The extracellular and intracellular MCP-1 protein level was assessed by ELISA and Western blotting methods, respectively. Abbreviations: N, normal group; M, model group; V, valsartan group. $^{\#}$, $p < 0.05$; $^{\#\#}$, $p < 0.01$; **, $p < 0.01$.

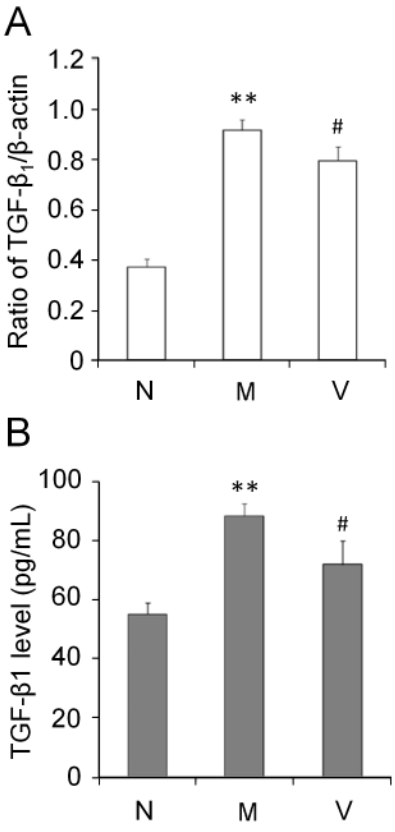

Figure 7. Effects of high-glucose levels and valsartan treatment on TGF-β_1 mRNA (A) and protein (C) expressions in GMCs. Abbreviations: N, normal group; M, model group; V, valsartan group. $^{\#}$, $p < 0.05$; **, $p < 0.01$.

4. Discussion

In the present study, we investigated the mechanisms underlying the renoprotective effects of valsartan in GMCs and GECs *in vitro*. Valsartan significantly reduced the production of ROS, increased levels of antioxidant agent GSH and antioxidant enzyme SOD, thus decreasing cell oxidative damage in GMCs and GECs induced by high-glucose levels. Valsartan also obviously suppressed the expression of proinflammatory cytokine MCP-1 in GMCs and GECs both at the protein and mRNA levels, which were both dramatically increased when cells were cultured in high-glucose conditions. In addition, the increased TGF-β_1 expression in GMCs induced by high-glucose levels was inhibited by valsartan treatment. These results suggest that the renoprotective effects of valsartan were possibly related with attenuating oxidative stress, decreasing the expression of MCP-1 and TGF-β_1 in GMCs and GECs.

Among the factors that induce pathological injury of glomeruli in the background of the diabetic milieu, the role of oxidative stress is supported by the observations that antioxidants suppress high-glucose induced ECM protein synthesis in mesangial cells and prevent glomerular and renal hypertrophy, albuminuria, and glomerular expression of ECM in experimental diabetic animals (*18-19*). Previous studies indicated

that RAS contributes to increasing cell oxidative stress (*20*). Kidney cells such as GMCs and GECs are able to synthesize all of the components of RAS such as renin, the (pro)renin receptor, angiotensinogen, and Ang II receptors independently of the systemic RAS, thereby making the kidney capable of maintaining a high level of local Ang II (*21*). Hyperglycemia may activate the intrarenal RAS, leading to accumulation of Ang II and activation of AT1 receptormediated signaling pathway in the kidney (*22-23*). Portero-Otin and colleagues demonstrated the inhibition of RAS decreases renal protein oxidative damage in diabetic rats (*24*). These results are consistent with our results and may suggest that valsartan decreases oxidative stress and improves oxidative damage to GMCs and GECs cultured in high-glucose conditions through blocking AT1 receptormediated signal transduction.

Our results showed that high-glucose levels increased the production of ROS in GMCs and GECs. Studies indicated that ROS may act as integral signaling molecules in diabetic nephropathy and its activation of protein kinase C (PKC) and the subsequent mitogen-activated protein kinases (MAPKs) play a critical role in high-glucose induced renal injury (*25-28*). PKC activation increases the expression of TGF-β, which causes an increase in mesangial matrix deposition and GBM thickening and may promote GECs apoptosis or detachment (*28-29*). In addition, high-glucose could induce MCP-1 synthesis by a PKC-dependent pathway. Since MCP-1 is the strongest known chemotactic factor for monocytes, its over-production would result in increasing monocyte immigration and monocyte activity and exacerbating interstitial fibrosis, thus worsening renal function. In the current study, addition of valsrtan decreased the production of ROS in GMCs and GECs induced by high-glucose levels. Thus, lessened activation of the PKC-MAPK pathway might contribute to down-regulation of TGF-β1 and MCP-1 expression in GMCs and GECs.

In conclusion, the current data demonstrated that valsartan efficiently decreased oxidative stress, TGF-β_1 and MCP-1 expression in GMCs and GECs cultured in high-glucose conditions. These mechanisms might be related with the renoprotective effects of valsartan.

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Association of salivary cortisol with chronomics of 24 hours ambulatory blood pressure/heart rate among night shift workers

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Summary **Recent studies indicate a circadian rhythm in blood pressure and heart rate and its association with various neurotransmitters. In the present study, we examine the circadian nature of blood pressure/heart rate and salivary cortisol in night shift workers and whether these circadian changes produced by night shifts are reversible. Sixteen healthy nurses of both genders, aged 20-40 years, performing day and night shift duties, were randomly selected out of 22 who volunteered for this study. Ambulatory blood pressure monitoring was done in all the subjects and salivary cortisol levels were analyzed during both day and night shift duties. There were clinically significant changes in the Acrophase of blood pressure and cortisol levels, indicating ecphasia (odd timing of systolic blood pressure) individually during night as well as day shifts. However, this pattern was statistically not significant. A reverse pattern of Acrophase was observed in 8 out of 16 subjects when they were posted on day shift. No significant change was found in midline estimating statistics of rhythm (MESOR) of blood pressure values. Changes in Double amplitude (Predictable change) were observed in 8 subjects during night shifts as well as in 7 subjects during day shifts. However, the pattern was not similar and night workers had an altered circadian pattern in the night as well as during day shifts. Changes in Double amplitude, Acrophase and Salivary cortisol were found during night as well as day shifts but these changes were not statistically significant ($p > 0.05$) due to incomplete recovery during day shifts (changes again seen when they came back to day shifts). Salivary cortisol levels were lowest in early morning, increased at midnight and further increased in the afternoon during night shifts along with ecphasia. It is possible that nurses working the night shift felt more tired due to the altered circadian cycle.**

Keywords: Rotating night shift, ambulatory blood pressure and heart rate monitoring, circadian cycle, ecphasia

1. Introduction

Night shift working may be associated with disruption of circadian rhythm, where a person's internal body clock is in swing with the shift schedule. The circadian rhythm of our body is characterized with an alternating cycle of sleep and awakening (*1*). Among healthy subjects, sleep tends to occur during a particular phase of the circadian cycle (*2*). Those who work during the night shift may attempt to sleep when their body clock is adjusted for the awakening phase (*3*). This attempt disturbs the body clock resulting in a contradictory relationship between sleep time and circadian schedule. There is evidence that shift work affects both sleep and awakening by disrupting circadian regulation which has adverse effects on family and societal life (*4*). The

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night shift work alters both length and quality of sleep. Day sleep is light, fragmented, and more likely to be disrupted and hence, insomnia can be severe in night shift workers (*5*). It is possible that circadian sleep propensity rhythm and hormonal rhythm are under influence of the circadian pacemaker as well as sleep habits (*6*).

Most rhythms are driven by an internal biological clock located in the hypothalamic suprachiasmatic nucleus and can be synchronized by external signals such as light-dark cycles (*7*). The rapidly rotating shift system including two consecutive night shifts, do not significantly alter the normal circadian rhythm of the body, particularly performance level, body temperature and hormone release (*8*).

The majority of the circadian rhythms in our body have both an endogenous component regulated by internal clock, Suprachiasmatic nuclei (SCN), and an exogenous component composed of a light-dark cycle (*1*,*5*). The disruption in the natural time pattern, under influence of a light-dark cycle, acts upon the circadian system to bring it into synchronization with the new time pattern. The circadian blood pressure variation is determined largely by the sleep and awakening cycle under influence of the internal body clock (*9*). Cortisol, a reliable indicator of stress, displays pronounced variation across the time-of-the day with high levels in the morning and low around midnight (*10*). Stress may alter intensity of secretion of cortisol and circadian pattern of the hormone. It is known that a long term increase of circulating cortisol or changes in the circadian rhythm of the hormone enhances the risk of metabolic and cardiovascular diseases including cancer, diabetes and depression (*11*). Identical heart rates and blood pressures have been observed among nurses working night shifts (*12*).

In the present study, we evaluated the circadian nature of blood pressure, heart rate and salivary cortisol in night shift workers, to find out if there is a relationship between circadian rhythm of blood pressure, heart rate and salivary cortisol levels and whether these changes are reversible after change in duty schedules.

2. Methods

2.1. *Subjects*

Out of 22 volunteers, 6 were excluded due to non-fulfillment of study protocol. The duration and pattern of shift work were the same among all the subjects. Sixteen healthy Healthy nursing professionals (Table 1), aged 20-40 years, performing day and night shift duties (continuous 9 days night shifts with alternate day shifts) for 8 years were willing for compliance to be randomly selected and recruited from Trauma Center, GM and Associated Hospitals, Chhatrapati Shahuji Maharaj Medical University, Lucknow, UP, India. The study was conducted from March to July, 2009 when the average temperature of the city ranged between 34°C and 38°C. At 26.50 N°, Lucknow is located just north of the tropic of cancer. All subjects were working in centralized air-conditioned wards. The study was approved by the institutional ethics committee (*Ref.* code: XXXIV ECM/B-P3) and written, informed consent was obtained from all subjects participating in the study. Healthy nursing professionals of both genders, aged between 20-40 years who performed night and day duty were included in this study. Subjects with any acute/chronic illness, known patients with diabetes mellitus, other endocrinal disorders, hypertension, coronary artery disease, and chronic renal diseases were excluded from this study.

Table 1. Height, weight and age distribution of male and female night shift workers

Baseline characteristics	Male (n = 8)	Female (n = 8)
Age	22.25 ± 1.28	26.50 ± 5.80
Weight (kg)	58.50 ± 10.53	50.12 ± 6.66
Height (cm)	164.50 ± 8.12	152.37 ± 3.38
Body mass index (BMI)	21.63 ± 3.80	21.61 ± 2.96

Data are presented as means ± S.D. *n*, number of subjects.

2.2. *Twenty-four-hour ambulatory blood pressure and heart rate monitoring*

Blood pressure and heart rate were recorded by an ambulatory blood pressure monitor TM-2430 (A & D, Tokyo, Japan) that can measure repeated oscillatory blood pressure and heart rate at desired intervals. Taking serial measurements a few times each day is important to reduce error associated with single measurement. The chronobiologic characterization of the circadian amplitude and Acrophase in addition to the midline estimating statistics of rhythm (MESOR) further reduces the error. Taking only one or two measurements a day, always at awakening and/or at bedtime may fail to reveal abnormalities seen only at other times of the day, or abnormalities that apply only to the variability in blood pressure or heart rate (*13*).

In this study, the subjects wore an ambulatory blood pressure monitor TM-2430 programmed to automatically measure blood pressure and heart rate at 30 min intervals while awake and sleeping hours during night shifts and again when they were shifted to day duties. The data were downloaded after every monitoring span to a local PC *via* an interface (TM-2421, A & D). Each blood pressure and heart rate profile was analyzed by a sphygmochron, utilizing both a parametric and non-parametric approach. Ambulatory blood pressure monitoring records were sent to Halberg Chronobiology Center, University of Minnesota, Minneapolis, MN, USA for further interpretation. Original oscillometric data from each blood pressure series was first synchronized according to the rest

activity cycle of each individual by recomputing all the records in hours, from bedtime to avoid differences among subjects in actual time of daily activity and to express results in circadian time rather than in less meaningful clock hours. After synchronization, blood pressure and heart rate values were edited according to commonly used criteria for the removal of outliers and measurement errors. The remaining data were analyzed chronobiologically.

The study of human chronomes can serve the derivation of refined reference values to better define health and to identify pre-disease, so that prophylactic intervention can be instituted as early as possible, preferably before disease sets in (*14*). In the current implementation of the chronobiological recommendations, reference values have been specified for clinically healthy peers of a given gender and ethnicity in different age groups (*15*). Ambulatory blood pressure monitoring was done during their day and night shifts. Some essential parameters which are directly under the influence of night shift work such as body temperature, time of arousal, time of going to bed, duration of nocturnal and diurnal sleep, mode of waking up, sleep latency, quality of sleep, feeding habits, menstrual history (for females), and family history were also recorded. Acrophase (hr:min, Time of overall high/peak values, and Hyperbaric index) were calculated for Systolic blood pressure (SBP), Diastolic blood pressure (DBP), and Heart rate. The circadian amplitude, a measure of the extent of reproducible variability within a day, was obtained by linear curve fitting, which yields added parameters: in midline-estimating statistics of rhythm, the MESOR (a time structure or chronome-adjusted mean), Acrophase (the timing of overall high values), recurring in each cycle, and the amplitude and Acrophase of the 12 hour (and higher order) harmonics of the circadian variation that with the characteristics of the fundamental 24 hour component, describe the circadian wave form. The MESOR is a more precise and more accurate estimate of location than the arithmetic mean (*15*).

2.3. *Estimation of circadian pattern of salivary cortisol levels*

Saliva samples were also collected at the time of ambulatory blood pressure monitoring. We collected saliva samples at approx. eight hours intervals in their night shift schedule (afternoon sample: 13:00 to 15:00, night samples between 22:00 to 01:00 and morning samples between 05:00 to 08:00) and during their day shift, 1st sample was taken between 14:00 to 15:00, 2nd at 21:00 to 22:00, and the last sample around 05:00 to 06:00 hours. The volunteers themselves collected the samples in different colored vials. For collection of saliva samples, a notebook was provided to each subject with all details regarding the timing and procedure for sampling and their sleep-wake timing. A thermometer was also given for recording of the circadian pattern of body temp. Each participant was instructed to wash their hands properly before taking the samples and to rinse their mouth with water to remove food particles, if they had taken their meals. They were asked to refrain from eating or drinking anything for at least 30 min after awakening. Saliva samples were then centrifuged at 3,000 rpm for 15 min. Cortisol samples were analyzed by the ELISA method. Salivary cortisol was estimated due to its stability in saliva for a longer time period and its ease of taking for circadian studies. The salivary cortisol concentration was synchronous with the serum concentration, indicating that the salivary assay could be substituted for the serum assay to assess circulatory rhythmicity across the 24-h time frame. Salivary cortisol appears to represent serum cortisol across the 24 h period, except for those on oral contraceptives (*16*). The more pronounced cortisol responses in saliva than in serum and its closer correlation with adreno-corticotropic hormone offer advantages over serum cortisol suggesting salivary cortisol measurement may be used as an alternative parameter in dynamic endocrine tests (*17*).

3. Results

As shown in Table 2, blood pressure and heart rate increased during the night and decreased in the early morning during night shift work. Alteration in mean Acrophase (time of overall peak value) of SBP in individual subjects during night shifts was observed, showing ecphasia (odd time of SBP, not of DBP and heart rate). The day shift was associated with a typical circadian rhythm with a drop in both SBP and DBP at night. This pattern was reversed in night shifts. Acrophase was found to be altered in 15 out of 16 subjects when they were working night shifts. This indicated that ecphasia (odd timing of blood pressure) was found in 15 subjects during night shifts. A reverse pattern of Acrophase was found in 8 subjects out of 16 when they were posted on day shifts. Chronobiological studies need to analyze the data individually not statistically. Changes in double amplitude, acrophase, and cortisol levels were significant clinically but these changes did not reach statistical significance due to incomplete recovery when subjects came back to day shifts.

Changes in double amplitude, Acrophase, and salivary cortisol were found during night as well as day shifts but those changes were not statistically significant ($p > 0.05$) due to incomplete recovery during day shifts (changes again seen when they come back to day shifts). No significant change was observed in MESOR. Alterations in MESOR values were observed in 2 subjects during night shifts and these altered patterns were reversed when they were changed to day

Table 2. Anti-HBV response of TCM and related active compounds in clinical trials

Parameters	During night shift	During day shift	*p* values
MESOR			
SBP	114.46 ± 9.32	113.31 ± 9.23	0.30
DBP	71.28 ± 6.73	70.42 ± 5.96	0.34
HR	73.87 ± 3.83	73.77 ± 4.04	0.46
Double amplitude			
SBP	22.48 ± 13.57	25.91 ± 13.36	0.25
DBP	17.55 ± 8.90	20.29 ± 8.66	0.15
HR	13.31 ± 7.57	15.80 ± 10.27	0.23
Salivary cortisol levels			
Evening	2.73 ± 1.90	3.03 ± 2.05	0.33
Night	3.34 ± 3.36	2.27 ± 1.95	0.13
Morning	3.46 ± 2.90	4.65 ± 2.83	0.09

Data are presented as means ± S.D.; Abbreviations: MESOR, midline estimating statistics of rhythm; SBP, systolic blood pressure; DBP, diastolic blood pressure.

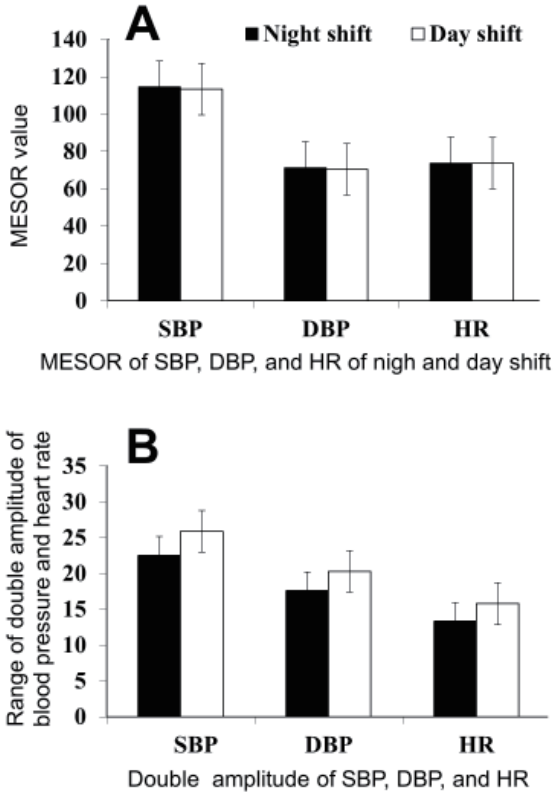

Figure 1. MESOR and Double amplitude of SBP, DBP, and Heart rate during night and dayshifts. (A) MESOR of night and day shifts. **(B)** Double amplitude of night and day shifts. Closed column, night shift; open column, day shift. Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate.

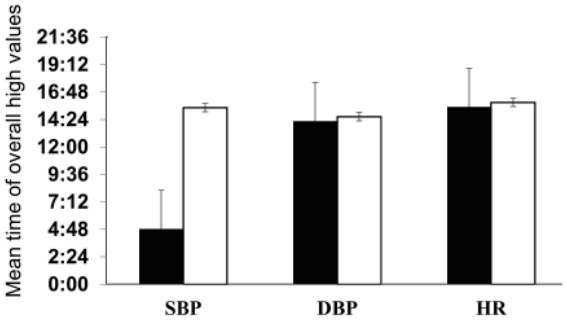

Figure 2. Acrophase (time of overall peak values) of SBP, DBP, and heart rate during night and day shifts. Closed column, night shift; open column, day shift. Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate.

shifts. Therefore, MESOR values were normal during day as well as during night shifts (Figure 1A). Changes in double amplitude were observed in 8 subjects during night shifts. While during day shifts, these changes were observed in 7 subjects only, but these patterns were not similar to that found during night shifts. A change in the pattern of double amplitude of SBP, DBP, and heart rate develops later on, 4-6 days after day shifts (Figure 1B).

Alteration in mean Acrophase (time of overall peak value) of SBP during night shifts was observed, showing ecphasia (odd time of SBP not of DBP and heart rate), which was reversed during day shifts (Figure 2). Significant changes were observed in Acrophase, showing ecphasia which may be a clinically significant cause of drowsiness, fatigue, and sleep disturbances in night shift workers. Hyperbaric index is the threshold or upper limit of the tolerance interval. It is a 3-h fractionated time interval. Alteration in circadian pattern of the hyperbaric index was observed during night shift due to an altered sleep-wake pattern, however, in day shift 3 subjects showed a reverse pattern (normal pattern) represented by NC (no change from reference range) which has been shown in Figure 3.

Salivary cortisol levels were decreased in early morning (in 5 subjects), increased at midnight (in 8 subjects) and were highest in the afternoon (in 8 subjects) during night shifts along with ecphasia (odd timing of blood pressure), while during day shifts the altered circadian pattern of cortisol was found to be different in subjects having a normal circadian pattern during night shifts (Figure 4). The normal circadian pattern of cortisol showed diurnal variation and decreased at night with an increase during early

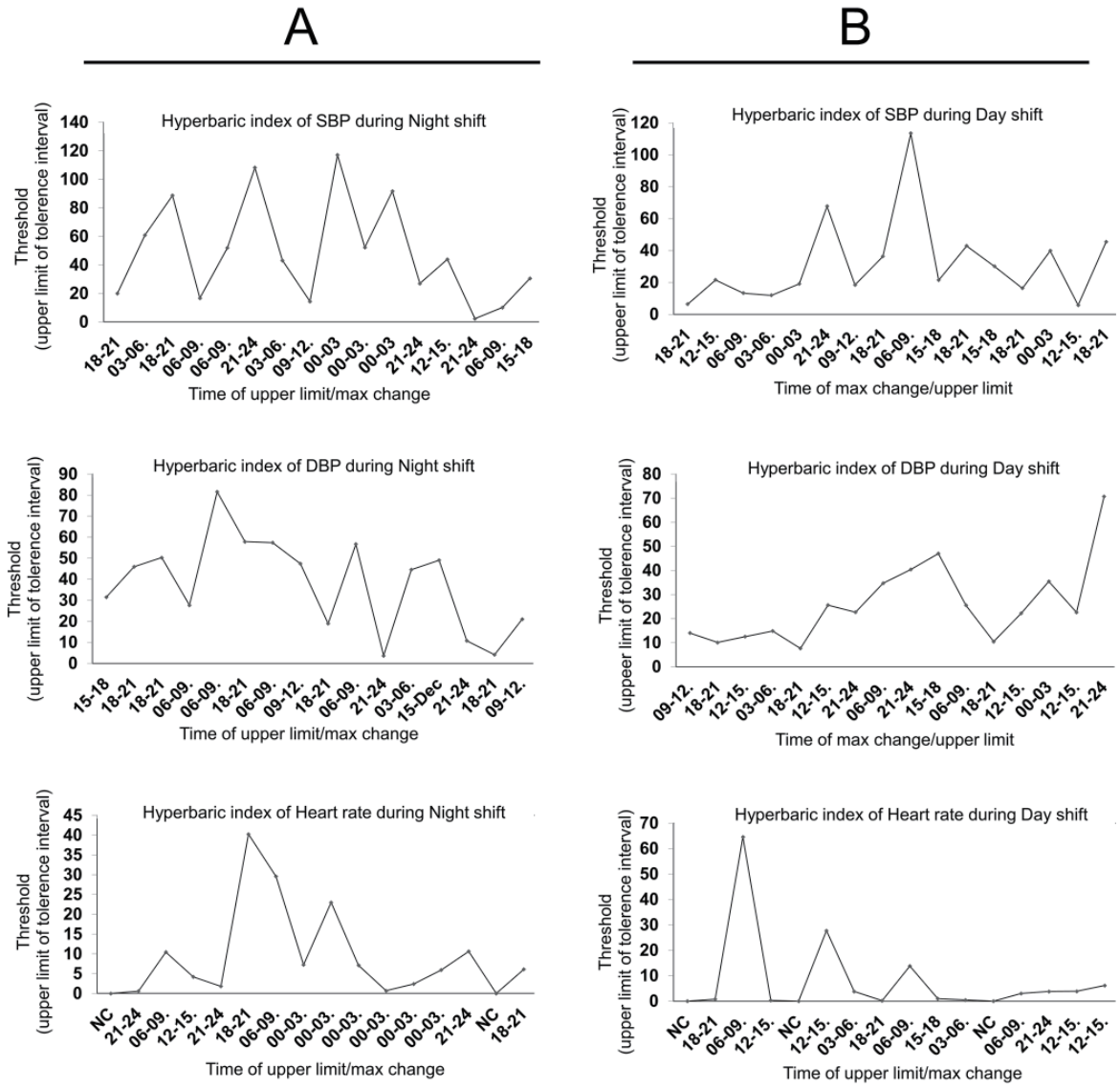

Figure 3. Hyperbaric Index of SBP, DBP, and Heart rate during night and day shifts. (A) Night shift. **(B)** Day shift. X-axis represents three hours fractionated time interval at which the maximum change found in a 24 h period. NC means no change from reference range. Y-axis represents threshold or upper limit of tolerance interval in a 24 h period.

morning. Evening cortisol levels during night shifts was normal, however; during day shifts, it showed a slight increase which suggests that the alteration in circadian pattern develops later when shifts were rotated. Night cortisol levels during night shifts were increased and a slightlyreversed pattern was found during day shifts which was not significantly reversed (Figure 5). Morning cortisol levels also showed a slightly reversed pattern during day shifts.

4. Discussion

Night shift workers are awake when they are supposed to sleep and attempt to sleep in day time when they are normally supposed to be awake. They have a higher incidence of poorer sleep and its complications (*18-20*). This study shows that the majority of subjects complained of headache, drowsiness, fatigue, and inadequate or poor quality of sleep because of difficulty in falling asleep and maintaining sleep. Our study shows an alteration in circadian pattern of Acrophase and double amplitude of blood pressure and heart rate during night as well as day shifts, which indicates, the phenomenon of desynchronization instead of resynchronization when they are reversed in day shifts. Changes in Acrophase (time of overall peak value) of SBP shows ecphasia (odd timing of SBP) during night shifts and this pattern was reversed during day shifts. A few studies have demonstrated that shift work is associated with increased cardiovascular morbidity and mortality (*21-23*). Alterations in Acrophase and double amplitude showed the predictive cardiovascular disorder. Increased frequency of blood pressure variations, in addition to high blood pressure, has been

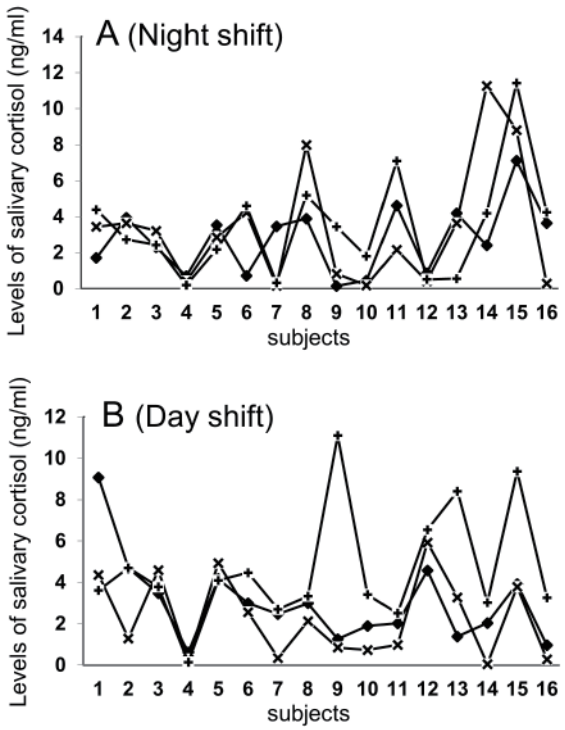

Figure 4. Circadian pattern of salivary cortisol levels during night and day shifts. (A) Night shift. **(B)** Day shift. ×, Night cortisol levels; +, morning cortisol levels; ♦, evening cortisol levels.

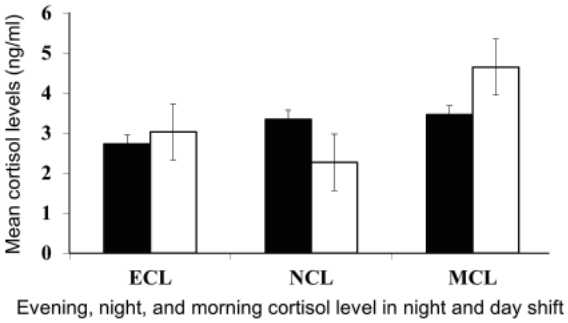

Figure 5. Circadian pattern of mean salivary cortisol levels during night and day shifts. ECL, evening cortisol level; NCL, night cortisol level; MCL, morning cortisol level. Closed column, night shift; open column, day shift.

associated with greater target organ damage and higher incidence of cardiovascular events.

The most important physiological mechanisms regarding shift work, particularly night shift work, is the problem of entrainment (resynchronization) of physiological functions after a phase shift of working and sleeping times (*19*). The internal desynchronization of circadian rhythm in physiological rhythms like oral temperature and grip strength are in favor of the hypothesis of an internal desynchronization and clinical intolerance to shift work (*20*). Physical activity is one of the determinants of Ambulatory blood pressure and diurnal variation (*24*,*25*).

Salivary cortisol appears to be an excellent measure for monitoring circadian rhythm variation in adrenal activity in healthy individuals during shift work (*26*). Higher salivary cortisol during morning and night shifts and the worst quality of sleep in engineers working very fast backward-rotating shifts may be an indication for insufficient recovery (*27*). A reversal of circadian function could be observed for the total group (mean cortisol concentrations) after the fifth night. They exhibited lower duration of less consistency in recovery of sleep across the following days after night work (*28*). The circadian patterns of cortisol during night shift were altered in the afternoon, night and morning phase from that of the normal pattern. The higher salivary cortisol in evening and night hours during night shifts and worse quality of sleep may be an indication for insufficient recovery. The circadian pattern of blood pressure (Acrophase) and cortisol showed a definite correlation in night shift workers. This altered circadian pattern of salivary cortisol appears to be important because it is cortisol which augments various regulatory mechanisms involved with cardio-respiratory regulation including blood pressure and heart rate.

In conclusion, night shift workers appear to have an altered circadian pattern of Acrophase and a double amplitude of blood pressure and heart rate during night as well as during day shifts. These altered circadian changes persisted in most cases even when they were on day shift. However, alteration in cortisol level was observed during night shifts and that cortisol pattern was reversed slightly during day shifts. A larger study would be necessary to confirm these findings. The majority of the nurses working night shifts felt more tired after work due to an altered circadian pattern which indicates that fatigue can negatively influence health, quality of performance, safety and thus, patient care. A chronobiologically interlinked shift design may be important for normal physiological functioning of such professionals to avoid complications of awakening in the night.

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