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TOXICOLOGY OF SMOKELESS TOBACCO: IMPLICATIONS FOR IMMUNE, REPRODUCTIVE, AND CARDIOVASCULAR SYSTEMS

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The popularity of smokeless tobacco (ST), or noncombusted tobacco, usually placed within the mouth to be chewed, sucked, or swallowed, is growing rapidly and its prevalence of use is rising globally, due (in part) to greater convenience, as allowable cigarette smoking areas are rapidly decreasing, and increased social acceptability. Though data are limited, ST usage has been directly linked to a number of adverse health outcomes. The potential role that immune dysfunction, including dysregulation of immune cells and their components, may play in the progression of these adverse health outcomes is only just beginning to emerge. Evidence suggesting reproductive outcomes, such as perinatal mortality, preterm birth, and reduced sperm viability, also exists in conjunction with ST use. Cardiovascular health may also be impacted by ST use, resulting in increased blood pressure and endothelial dysfunction, both of which may potentially lead to cardiovascular diseases. This review describes the toxicological implications associated with ST use, with emphasis on immune, reproductive, and cardiovascular outcomes. Epidemiological studies are discussed with respect to experimental studies to help develop the relationship between ST and disease pathology. This review also summarizes the gaps in ST knowledge and potential future directions that are needed to more fully delineate the complex systems driving the adverse health outcomes associated with its use.

The tobacco plant has been smoked, powdered, chewed, or inhaled for hundreds of years. Documentation of its usage has been found dating back to use by Native Americans in the 15th century (Christen et al. 1982; IARC 2007). Smokeless tobacco (ST) is a general term that represents different forms of noncombusted tobacco, usually placed within the mouth to be chewed, sucked, or swallowed. The different forms are also dependent on supplemental additives used and physical form of the tobacco. Tobacco used for ST can be mixed with various spices and/or nut extracts and may come in powder or crystalline form, or even as moist or dry "leaves." The most commonly used ST variations include chewing tobacco (loose leaf, plugs, or twists), snuff (moist or dry, finely ground tobacco, placed between the gum and cheek or inhaled nasally), snus (moist, finely ground tobacco, contained in a teabag-like sachet, placed behind the upper lip without chewing), paan or betel quid (areca nut, slaked lime, spices, seeds, catechu—available with or without powdered tobacco, all wrapped in betel leaf), gutkha (powdered tobacco, areca nut, slake lime, spices, sweeteners, seeds, catechu—powdered and granulated in a foil packet), mishri (baked/burnt powdered tobacco), and naswar (powdered tobacco, slake lime, spices, cardamom, menthol) (Changrani and Gany 2005; IARC 2007; Rogers 2009).

The prevalence of ST use is increasing annually, and the type used is often dependent on regional preferences (Figure 1). For example, populations in the United States are

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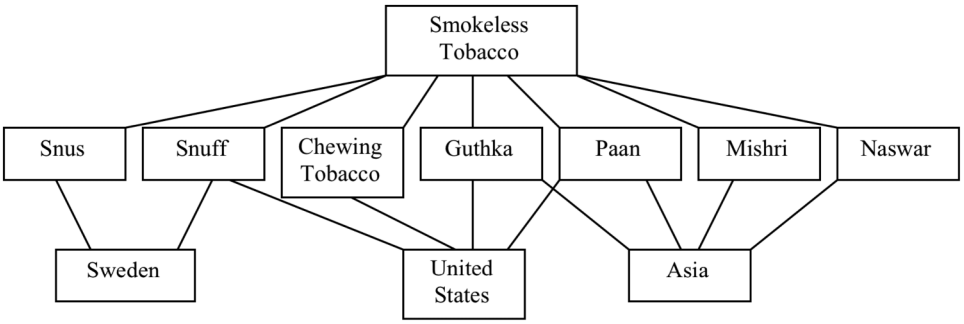

FIGURE 1. Flow chart of common forms of smokeless tobacco found in Sweden, the United States, and Asia.

most likely to use chewing tobacco or moist snuff, Swedish ST users employ snus, and South Asians use tobacco contained within paan, gutkha, mishri, and naswar (Changrani and Gany 2005; IARC 2007; Connolly and Alpert 2008; Javed et al. 2010). In Pakistan and India, there have been an estimated 100 million users of ST, with paan being the most popular form used in those regions (IUAC 1996). Moreover, in Bombay, India, of nearly 100,000 adults over 35 yr of age, over 32% reported chewing paan (Gupta 1996). The number of female ST users is rapidly increasing in many regions (Norberg et al. 2011), including among women of childbearing age (Sinha et al. 2011). In particular, use among women in South Asia appears to be becoming more common. For example, in the aforementioned study conducted in Bombay, it was estimated that almost 60% of the nearly 60,000 women surveyed reported using ST on a regular basis (Gupta 1996). Similarly, a study by Yang et al. (2001) examining the effect of betal quid on adverse birth outcomes among the Aborigine in eastern Taiwan found that out of the 229 subjects questioned, 118 (52%) reported usage of the product.

The prevalence of chewing tobacco, snuff, or snus use in the United States among individuals aged 18 yr or older was most recently examined in 2010 in the *Morbidity and Mortality Weekly Report* (MMWR), published by the Centers for Disease Control and Prevention (CDC). The study, which analyzed data from the 2009 Behavioral Risk Factor Surveillance System (BRFSS), reported the highest prevalence of ST usage by adults to be 9.1%, in Wyoming, while California ranked the lowest at 1.3% of all adults. Prevalence of use was greater among men than among women in all U.S. states. The prevalence of ST use in men was highest in West Virginia and lowest in the District of Columbia. Nationwide, usage of ST was inversely related to socioeconomic status and education level, and most prevalent among young adults aged 18 to 24 yr (CDC 2010); in contrast, ST use in South Asia is directly related to socioeconomic status (Arnab Mukherjea personal communication).

People reported using ST to achieve improved mental status and positive physiological manifestations, including improved concentration and relaxation, heightened alertness, diminished hunger, and improved digestion (Changrani and Gany 2005). It has also been used as a "gateway" to stop smoking (Melikian and Hoffmann 2009). In addition, it has been (1) deemed by some to be less harmful than cigarette smoking due to the lack of inhaled combustion by-products (Bates et al. 2003; Benowitz 2011; Colilla 2010, Foulds and Kozlowski 2007; Kozlowski 2002; Lee 2011); (2) thought by some to be more convenient, as allowable cigarette smoking areas are rapidly decreasing; and (3) considered by some more socially acceptable, in certain regions, than cigarette smoking (Melikian and Hoffmann 2009). In addition, mishri and some of the lesser known commercially manufactured ST (Denobac, Tona, or Ganesh) are used on the teeth and gums (primarily by women) in India to clean/whiten teeth (Gupta 2001; WHO 1997).

Smokeless tobacco contains >3000 constituents that contribute to the final nicotine content, pH, and taste of the tobacco (Hoffmann and Hoffmann 1997; IARC 2007; Roberts 1988). The exact components depend on the region in which the tobacco is grown, which pesticides are used, and the specific curing process used to treat the leaves (IARC 2007). Possible constituents include approximately 20 different *N*-nitrosamine compounds, 30 different pesticides, including chlordane, dieldrin, endrin, and/or heptachlor, and a variety of additives including menthol, benzyl benzoate, and/or eugenol (Hoffmann et al. 2001). Of these constituents, approximately 30 are known or suspected human carcinogens, including benzo[a]pyrene (BaP), urethane, some heavy metals, polonium-210, volatile organic compounds such as *N*-nitrosamines and aldehydes, and lactones, among others (Brunnemann and Hoffmann 1992; IARC 2007).

The various forms of ST contain differing amounts of nicotine and may vary among commercial brands. Chewing tobacco products, for example, have a nicotine content ranging from 3.4 to 39.7 mg/g product, while dry or moist snuff products range from 10.5 to 24.8 and from 4.7 to 24.3 mg/g product, respectively (Djordjevic and Doran 2009). Nicotine content in Swedish snus may range from 7.8 to 15.2 mg/g, while commercially available gutkha has a nicotine content ranging from 1.1 to 2.3 mg/g product (Stanfill et al. 2011). Comparatively, Djordjevic and Doran (2009) reported that the amount of nicotine contained within Phillip Morris's sample of international cigarette brands ranged from 13.79 to 23.18 mg/g product. Often the addition of alkaline modifiers in ST boosts pH, which may result in conversion to more rapidly adsorbed unionized nicotine (Tomar and Henningfield 1997), a characteristic more commonly observed in non-U.S. forms of smokeless tobacco (Djordjevic and Doran 2009; Stanfill et al. 2011).

A number of serious, adverse human health outcomes have been linked to ST use (reviewed in IARC 2007). These include periodontitis (Johnson and Slach 2001), oral leukoplakia, oral submucous fibrosis, gastrointestinal (GIT) abnormalities (Aro et al. 2010; Shankaran et al. 1994), oropharyngeal, esophageal, and pancreatic cancers (Boffetta et al. 2005; Johnson et al. 1994; Trivedy et al. 2002), and cancer of the stomach (Chao et al. 2002).

This review focuses on current existing knowledge concerning the adverse toxicological effects of ST, with particular emphasis on the immune, reproductive, and cardiovascular systems, as outlined in Table 1. While a number of epidemiological studies have been published concerning the potential health consequences of ST (Boffetta et al. 2008; Gupta and Subramoney 2006; Gupta et al. 2007; Henley et al. 2005; IARC 2007; Winn 1997), only a few of the more pertinent studies are described here to provide a context for selection of the ST-related toxicological outcomes. This review may help bring to light those in vivo and in vitro studies that provide plausibility and mechanistic explanations for associations observed in epidemiological studies. The particular organ systems selected for discussion herein include the immune, reproductive, and cardiovascular systems, selected because of their apparent link with ST usage. Moreover, these representative systems when impacted by a toxic chemical may potentially lead to serious disease outcomes associated with chronic health consequences. Whenever stated, the specific form of ST used for the cited study is identified. Scientific information used for this review was obtained from a thorough Medline and PubMed search, using terms including "smokeless tobacco," "snus," "gutkha," and "mishri," with "immune," "reproductive," "developmental," and "cardiovascular disease," to uncover studies performed over the last two decades; in a few cases, earlier studies are used to make a specific point. Future directions for ST research and major gaps in knowledge are also highlighted in this review. For additional information on human health outcomes associated with ST usage, the reader is referred to the IARC monograph on smokeless tobacco (2007), and selected

TABLE 1. Summary of the Toxicity of Smokeless Tobacco (ST) on the Immune System

Study design	Publication date	Authors	Tobacco type or surrogate used	Outcome
In vitro (cell systems)	1995	Bagchi et al.	ST extract	Cytotoxicity and apoptosis of macrophages
	2010	Lombard et al.		
	1997	Petro & Zhang		↓ IFN- γ
				↑IL-2
	2010	Johnson et al.	Nicotine	↑ IL-1
				↑ IL-8
Cross-sectional	2004	Shulman, et al.	ST[a]	Mucosal lesions are present in 60% of ST users
Observational	1982	Hirsch et al.	Snuff	Mucosal inflammation is associated with plasma extravasation and leukocyte infiltration
	1994	Johnson et al.		Elevated IL-1 in ST lesions
	1998	Payne et al.	ST[a]	Reduction in machrophages at ST placement sites

[a]No specific ST type provided.

epidemiological reviews, including, but not limited to Winn (2001), Rodu and Jansson (2004), and Lee and Hamling (2009).

IMMUNE TOXICOLOGY

The immune response is a delicately balanced, complex, and tightly-controlled system made up of lymphoid organs, cellular populations, and signaling mediators, all with a variety of functions (Luster et al. 1989). Immunoenhancement may result in exaggerated responses, which may potentially lead to hypersensitivity, resulting in increased inflammation and/or tissue damage (Luster and Rosenthal 1993). Immunosuppression, on the other hand, may lead to repeated, prolonged, and severe infections, as well as potential development of cancer (Luebke et al. 2004). During an inflammatory response, phagocytes present at the local site of tissue damage become activated and begin digesting and destroying the foreign pathogen (Soehnlein and Lindbom 2010). Once activated, these cells begin to produce cytokines that further activate other immune cells and stromal cells, such as endothelial cells and fibroblasts. Activated endothelial cells facilitate the response of circulating leukocytes into the area by releasing chemoattractants; effects of ST on these inflammatory mechanisms are described later.

Inflammation and Oral Mucosal Lesions

The relationship between ST use and diminished oral health has been well documented (Grady et al. 1990; Gupta et al. 2005; Phukan et al. 2002; Shulman et al. 2004). Although the form of ST used was not specified, Shulman et al. (2004) found that oral mucosal lesions are present in approximately 60% of current ST users in the Uniterd States, and its use is the strongest risk factor for the development of oral lesions and leukoplakia. Such ST-associated lesions are typically defined as (1) keratosis; (2) gingival inflammation, periodontal inflammation, and alveolar bone damage; (3) dental caries, tooth abrasion, and staining of tooth structure; and (4) dysplasia and oral cancer. Further epidemiological evidence of the correlation between ST and oral mucosal lesions was described by Kallischnigg et al. (2008). While the link between ST use and mucosal inflammation and oral cancer is well recognized (Chen et al. 2002), its effect on the human immune system and the role that ST plays as an immune mediator in relation to these outcomes are less clear.

Evidence for a role of the immune system in relation to adverse health impacts associated with ST use is growing. Typically, lesions and inflammation associated with ST use are found at the local site of placement within the oral cavity (Johnson and Slach 2001).

Gingival keratinocytes or epithelial cells are the first to come in contact with ST-associated chemicals. These cells produce cytokines, such as interleukin (IL)-1 and IL-8. The proinflammatory cytokine, IL-1, acts as to initiate inflammation, activate macrophages and osteoclasts, and increase the release of proteolytic enzymes. IL-1 also stimulates IL-8 secretion, known to recruit and activate neutrophils (a marker immune cell type of inflammation) in the surrounding tissue (Bickel 1993), in turn increasing periodontal destruction. Early studies by Hirsch et al. (1982) demonstrated that oral mucosa inflammation elicited by snuff use was characterized histologically by plasma extravasation along with leukocyte infiltration. In human subjects, IL-1 levels are elevated in habitual snuff placement sites compared to nonplacement sites in the same users (Johnson et al. 1994). These results provide evidence that ST components exert a localized effect on IL-1 production, which may in turn contribute to the observed local and/or systemic changes associated with ST use.

In vitro studies also support the role of IL-1 in ST-induced oral lesions. A recent study by Johnson and colleagues (2010) showed that the combination of lipopolysaccharide (LPS) and nicotine, an active ingredient of both smoked and ST, stimulated production of the highest levels of IL-1 and IL-8 in cultured gingival keratinocytes relative to either treatment alone. Offenbacher and Weathers (1985) suggested that bacterial factors may also play a role in the development of these lesions and coincide with the clinical observation that tobacco users with poor oral hygiene display more severe periodontal destruction than those with good oral hygiene.

One clinical study relating to ST-induced immunomodulation examined the possible role of macrophages and/or monocytes in the development of ST-induced oral lesions (Payne et al. 1998). CD11b+ macrophages and other phagocytic cells in the lamina propria, such as dendritic cells, play an important role in the development and resolution of oral inflammation by the release of anti-inflammatory cytokines and clearance of damaged or dead cells from the injured site (Guzik and Potempa 2008). It was observed clinically that early tissue changes and inflammation at the oral site of ST placement were associated with a significant reduction in the volume density of macrophages (particular subset not identified), which may potentially be associated with prolonged inflammation. Histological changes in oral tissue were also apparent in the user as early as 2 d following the placement of ST (Payne et al. 1998). Thus, disruption of macrophage levels may exert significant effects on oral mucosal inflammation and lesions associated with ST use.

In vitro data support the clinical observations of macrophage depletion associated with ST use. Bagchi et al. (1995) demonstrated that exposure of a cultured macrophage cell line (J774A.1) to ST extract led to cell cytotoxicity and apoptosis. Bagchi et al. (1995) reported that these effects occurred via the induction of oxidative stress, as free radical scavengers and several antioxidants were able to inhibit ST-induced apoptosis of these cultured cells.

Cellular damage due to oxidative stress following exposure to ST is not exclusive to effects on macrophages. Smokeless tobacco was shown to produce oxidative cell damage and apoptosis in a concentration-dependent manner in human oral kerotinocytes, which could be attenuated by treatment with antioxidants (Bagchi et al. 1999). Lombard et al. (2010) recently demonstrated that exposure of another macrophage/monocyte cell line (MM6) to increasing concentrations of ST extract also led to a concentration-related decrease in cell viability and enhanced apoptosis.

Experimental studies by Petro and Zhang (1997) showed that T-lymphocyte activity and cytokine production were also affected by ST exposure. For these studies, whole murine splenic mononuclear cell populations and enriched T-lymphocytes costimulated with anti-CD3 and anti-CD28 antibodies were examined. Enriched T-lymphocytes, exposed in vitro to increasing dilutions of ST extract, demonstrated reduced levels of interferon (INF)-γ and IL-10, while IL-2 production

was raised compared to nonextract treated cells. As IL-10 acts to inhibit the proinflammatory effects of IL-2 and elevated levels of IL-2 were reported to be an important feature of periodontal disease (McFarlane and Meikle 1991), a decrease in IL-10 may exert a negative health impact on the control of inflammation in relation to periodontal disease. Further, as IFN-γ is critical for activating immune cells, an ST-induced fall might impact the progression of microbial infection, and possibly the development/progression of oral cancer.

REPRODUCTIVE TOXICOLOGY

Perinatal Mortality, Preterm Birth, and Preeclampsia

As previously stated, global use of ST products is by no means exclusive to men. In fact, ST use among women was reported to be as high as 60% in several regions of India (Norberg et al. 2011; Rani et al. 2003; Sinha et al. 2002). In addition, ST usage among Swedish women, particularly of childbearing age, has also been on a steady rise. For example, Norberg et al. (2011) reported that >6% of 30-yr-old women began using snus between 2002 and 2007.

Table 2 summarizes the toxicity of ST on the reproductive system. Rapidly accumulating epidemiological evidence suggests a "reason to worry" about the reproductive/developmental risks associated with ST (England et al. 2003; Gupta and Supramoney 2004; 2006; Said et al. 2005; Wikstrom et al. 2010a; 2010b; 2010c; Yang et al. 2001). Preterm birth and lowered birth weight appear to be associated with prenatal ST use. For example, a study of Mumbai women in their second and third trimesters found birth weight reductions of >100 g in ST users (Gupta and Subramoney 2004) and an odds ratio (OR) for low birth weight of 1.6 (1.1 to 2.4) in newborns whose mothers used ST during pregnancy compared to controls. In the same study, gestational duration of maternal ST users was reduced by 6.2 d. Common forms of tobacco use reported in the study were mishri, and paan with tobacco (gutkha). In a population-based study of pregnant women in Sweden, England et al. (2003) reported that snuff users had a significantly increased incidence of preterm birth, with an adjusted OR of 1.98 (1.46–2.68); relative risk for snuff users was even greater than that for cigarette smokers (adjusted OR = 1.57 [1.38–1.80]). England et a.l (2003)

TABLE 2. Summary of the Toxicity of Smokeless Tobacco (ST) on Reproductive System

Study design	Publication date	Authors	Tobacco type or surrogate Used	Outcome
In vivo (animal models)	2001	Aydos et al.	Nicotine	Structural alterations in testes
	2011	Archana et al.	Paan masala	↓ Female fertility
				↓ Gestational duration
				↓ Birth weight
				↑ Risk of neonatal death
Cohort	2003	England et al.	Snuff	↑ Risk of preterm birth
				↑ Risk of preeclampsia
	2004	Gupta and Subramoney	Mishri, paan with tobacco, gutkha	↓ Birth weight
				↓ Gestational duration
	2005	Said et al.	ST[a]	↓ Sperm concentration, motility, morphology, percent viability
	2006	Gupta and Subramoney	Mishri, gutkha	↑ Incidence of stillbirth
				↑ Risk of preterm birth
	2010	Wikstrom et al.	Snus	↑ Risk of preterm birth
	2010	Wikstrom et al.		↑ Risk of stillbirth
	2010	Wikstrom et al.		↑ Risk of preeclampsia
Case-control	2001	Yang et al.	Betel quid	↓ Birth weight
				↑ Risk of preterm birth

[a]No specific ST type provided

speculated that this finding may be associated with the observation that while snuff use increased hypertension during pregnancy (a risk factor for preterm birth), cigarette smoking was shown to be protective of this outcome—an observation that previously documented by Conde-Agudelo et al. (1999), although the exact mechanism(s) is currently unknown. More recently, Wikstrom et al. (2010c) demonstrated that snus users had elevated risks of very (<32 gestational weeks) and moderate (32–36 gestational weeks) preterm birth. A similar finding was observed in a study by Yang et al. (2001) examining the association between betel quid chewing and adverse birth outcomes among Aborigines in eastern Taiwan. Betel quid chewing during pregnancy in this population resulted in a significant decrease in birth weight and an increase in preterm birth.

The risk of stillbirth also appears to be affected by ST usage. A population-based prospective cohort study in Mumbai showed a significantly higher incidence of stillbirth (adjusted OR = 2.6 [1.4–4.8]) in women who used mishri or gutkha during pregnancy compared to nontobacco users. The risk of stillbirth in these women was dependent upon ST frequency and specific time periods during gestation when ST was used (Gupta and Subramoney 2006). Similarly, a large population-based study demonstrated that Swedish snus users had a significantly higher risk of stillbirth and preterm birth than nontobacco users. Risk for both of these obstetric outcomes was dose dependent; the risk of stillbirth was elevated in snus users compared to light cigarette smokers (i.e., 1–9 cigarettes/d) and numerically lower than that observed for heavy smokers (i.e., >9 cigarettes/d) (Wikstrom et al. 2010a). These finding are alarming, considering the growing use of ST products by women of childbearing age. While the epidemiological data clearly suggest an association between ST usage and adverse obstetric outcomes, toxicological studies to define the feasibility and underlying mechanisms of such relationships have yet to be performed.

As previously mentioned, unlike that observed with cigarette smoking (Conde-Agudelo et al. [1999] reported a reduced risk of preeclampsia in smokers), studies by England et al. (2003) and Wikstrom et al. (2010c) associated ST usage (specifically, snuff and snus, respectively) with increased risk of preeclampsia. Although this is not fully understood, England et al. (2003) and Wikstrom et al. (2010b) speculated that such dichotomy between cigarette smoking and ST may be due to an unknown product of smoke combustion that serves as a protective factor for preeclampsia. In the absence of this protective factor, nicotine might potentially raise the risk of preeclampsia via effects on the cardiovascular system, perhaps by producing endothelial dysfunction or raising blood pressure.

Although extremely limited, animal studies support the link between ST usage and detrimental obstetric outcomes. In a recent study by Archana et al. (2011), pregnant mice were exposed to tobacco-associated pan masala with tobacco from gestation day 0 until lactation. The authors found that exposure to pan masala with tobacco significantly reduced gestational length and birth weight in mice. A reduction in female fertility was also observed, as well as increases in post-implantation loss and neonatal death with respect to controls.

Reproductive risks associated with ST are not exclusive to females (Said et al. 2005). A study at an infertility clinic in India reported that among men who used unspecified ST, the concentration, motility, morphology, and percent viability of sperm were all significantly lower for those men who were severe users (>6 times/d) compared to heavy ST users (3–6 times/d); effects on fertility were greater for heavy users than those who used ST <3 times/d. Murine studies, using nicotine as a surrogate for ST, provide mechanistic insight for these findings. Injection of mice with nicotine revealed several significant ultrastructural alterations in the testes (compared to control animals) that included thickening of the tunica propria and an increase in collagen fibers and a degeneration of the intercellular tight junctions between Sertoli cells and germ cells (Aydos et al. 2001). The same investigators also reported morphological changes

in spermitids themselves following nicotine exposure. Thus, nicotine derived from either smoked or smokeless products can produce Sertoli cell and/or spermatatid dysfunction critical for spermatogenesis.

CARDIOVASCULAR DISEASE

Blood Pressure and Heart Rate

Table 3 summarizes the toxicity of ST on the cardiovascular system. Epidemiological evidence exists that suggests an association between ST use and both immediate and long-term increases in blood pressure and heart rate (Bolinder et al. 1992; Gupta et al. 2007; Pandey et al. 2009). In a population-based, cross-sectional study of construction workers in Sweden, ST users were more likely than nonusers to have a diastolic blood pressure >90 mm Hg (OR = 1.8 [1.5–2.1]) and a systolic blood pressure >160 mm Hg (OR = 1.7 [1.3–2.1]) (Bolinder et al. 1992). A link between ST use and high blood pressure was also reported in adult males living in India (Pandey et al. 2009). Specifically, mean systolic and diastolic blood pressure was significantly higher in users of snuff, chewing tobacco, or paan containing tobacco compared to nonusers; the incidence of diastolic hypertension was also elevated in this study (OR = 2.3 [1.3–4.3]). A relationship between ST use and high blood pressure is supported by a study in northwestern India that demonstrated a significant elevation in systolic and diastolic blood pressure, as well as resting heart rate compared to nonusers (Gupta et al. 2007).

Animal studies that examine ST as a possible mediator of hypertension and/or increased heart rate are rare. However, an early study by Squires et al. (1984) examined the effects of moist snuff (2.5 g) placed in the buccal cavity of anesthetized dogs for 20 min on a variety of hemodynamic parameters. The investigators noted significant increases in heart rate, blood pressure, left ventricular pressure, left ventricular end diastolic pressure, and dP/dt (first derivative of left ventricular pressure) in exposed animals.

TABLE 3. Summary of the Toxicity of Smokeless Tobacco (ST) on the Cardiovascular System

Study design	Publication date	Author	Tobacco type or surrogate used	Outcome
In Nitro	1996	Suzuki et al.	ST extract	Impairment of endothelial vasodilation
	2011	Laytragoon-Lewin et al.	Nicotine or extract	↑ DNA Synthesis in human endothelial cells and fibroblasts
In Vivo	1984	Squires et al.	Snuff	↑ Heart rate and blood pressure
Cohort	2002	Accort et al.	ST[a]	No association with CVD mortality
	2005	Henley et al.	Chewing tobacco and snuff	↑ Mortality from all CVD
	2007	Hergens, et al.	Snuff	Increased risk of fatal MI
Case-Control	1992	Siegel et al.	Chewing tobacco and snuff	No difference in systolic and diastolic blood pressure in athletes
	2007	Gupta et al.	ST[a]	↑ Diastolic and systolic blood pressure ↑ Resting heart rate
	1991	Eliasson et al.	Snuff	No significant change in diastolic blood pressure
	2003	Asplund et al.		No significant change in stroke
Cross-sectional	1992	Bolinder et al.	ST[a]	↑ Diastolic and systolic blood pressure
	2001	Wallenfeldt et al.	Snuff	No significant effect on C-reactive protein levels
	2009	Pandey et al.	Snuff, chewing tobacco, and paan with tobacco	↑ Diastolic and systolic blood pressure
	2003	Grandbarry	Snuff	Endothelial dysfunction
Observational	2007	Haglund et al.	Snuff	No excess risk of mortality or hospitalization from stroke

[a]No specific ST type provided.

Alternatively, not all evidence points to an association between ST use and hypertension and/or increased heart rate (Eliasson et al. 1991; Siegel et al. 1992). In a study conducted on young professional baseball players, no significant differences in systolic or diastolic pressure, and/or pulse between snuff or chewing tobacco users and nonusers were observed (Siegel et al. 1992). A similar lack of effect was seen in a population study examining young snuff users in Sweden (Eliasson et al. 1991). In this case, no marked change in diastolic blood pressure was observed between ST users and nonusers. Chemical-induced effects on blood pressure and heart rate are multifactorial and are dependent on a variety of factors, such as age and physical activity, all of which make data interpretation in human studies difficult. Conflicting data between the epidemiological outcomes further emphasize the need for in vivo toxicological studies that employ a controlled exposure scenario with a single well-characterized type of ST.

Endothelial Dysfunction

Data are controversial as to whether ST usage leads to endothelial dysfunction, an important early event in atherosclerosis and in the progression of cardiovascular morbidity. A cross-sectional study conducted by Granberry et al. (2003) investigated whether usage of chronic moist snuff caused endothelial dysfunction, as measured in the brachial artery. In this case, blood flow increases were induced in the forearm of snuff users and cigarette smokers who refrained from tobacco use for 8 h before the procedure. Results of this study demonstrated that endothelial-dependent fiow-mediated dilatation (FMD) values were significantly impaired in both ST users (4.1 $\pm$ 0.7% dilation) and tobacco smokers (3.9 $\pm$ 5.1%) when compared with nontobacco users (12.2 $\pm$ 5.7%). Additional support linking ST with endothelial cell alterations comes from a recent in vitro study by Laytragoon-Lewin et al. (2011). In this case, primary adult human endothelial cells and fibroblasts, exposed to pure nicotine or nicotine-containing snus extract, demonstrated enhanced DNA synthesis in both treatment groups. Morphologic changes, not associated with cell death, were also observed in cells from both treatment groups, mostly in the form of prominent cytoplasmic vacuoles. The exact mechanism(s) underlying snus-induced endothelial changes in this model is not clear. However, as cellular proliferation and cellular morphologic changes were similar in both nicotine- and nicotine-containing snus-treated cells, it appears that nicotine may play a critical role in ST-induced endothelial dysfunction. In contrast to those studies demonstrating a possible link between ST and endothelial cell dysfunction, a study by Wallenfeldt et al. (2001) that examined 58-yr-old tobacco smokers, snuff users, and nonusers of tobacco revealed no apparent association between snuff use and carotid and femoral artery intima-medial thickness or with increased levels of C-reactive protein (CRP) as was observed in the tobacco smoking cohort.

Experimental studies examining the effects of ST on endothelial dysfunction are lacking. However, a study conducted by Suzuki et al. (1996) demonstrated that exposure of hamster cheek pouch oral mucosa to aqueous ST extract in situ produced impairment of agonist (acetylcholine and bradykinin)-induced endothelium-dependent vasodilation.

Cardiovascular Disease (CVD) and CVD Mortality

Epidemiological evidence demonstrating an increased risk from ST use on CVD is conflicting. While some ST studies demonstrate an association with CVD, CVD mortality, coronary heart disease, and stroke, others fail to support these claims.

Two prospective studies by Henley et al. (2005) analyzed the mortality rates of ST users from data obtained from a large U.S. cancer prevention study. Two cohorts (i.e., CPS-I and CPS-II) were analyzed. The first cohort included men enrolled in the study in 1959 with a 12-yr follow-up, while the second included men enrolled in a 1982 study with

an 18-yr follow-up. Taken together, these studies demonstrated that use of chewing tobacco and/or snuff in never-cigarette-smoking men was associated with significantly increased mortality from all CVD and stroke. In another study, Swedish construction workers demonstrated an elevated relative risk of fatal myocardial infarction associated with Swedish snuff use (Hergens et al. 2007). Heavy users, defined as those who used more than 50 g of snuff per day, had the greatest risk of fatal myocardial infarction. Mechanisms by which ST use may lead to increased risk of stroke and/myocardial infarction are currently unclear. In contrast, other epidemiological studies failed to find an association between ST use and elevated frequency of CVD mortality (Accortt et al. 2002; Asplund et al. 2003; Haglund et al. 2007). However, when considering such a serious long-term and financially draining disorder such as CVD, it is important to realize that for those regions with a high prevalence of ST use, even a small quantitative increase in relative risk could represent a large public health impact.

CONCLUSIONS AND KNOWLEDGE GAPS

Smokeless tobacco has existed for centuries and its use has followed a steady upward trend, particularly in North America and Northern Europe. Usage in developing nations, such as India and other Asian countries, is pervasive. ST continues to be popular among young adults and older children, worldwide (Page and Danielson 2011). The popularity of ST can be attributed to a number of factors, including unsupported perception of safety; perceived health advantages of ST use over smoked tobacco; banning of indoor smoking; ease and ability to use in a concealed manner; social acceptance; "positive" physiological effects; and a "way out" of smoking.

Smokeless tobacco is rapidly gaining social acceptance, yet research on the safety and potential adverse health outcomes is gravely lacking. To date, most tobacco research focuses primarily on the effects of smoked tobacco. Furthermore, any studies of "smokeless tobacco" tend to use surrogates of ST exposure, such as nicotine. Use of ST surrogates for safety assessments is highly problematic as there are likely to be key differences in chemical composition between ST and these other forms, as well as dissimilarities in the physical nature of exposure. It is therefore inappropriate to presume that findings from these surrogate studies can be used to extrapolate and accurately predict effects associated with ST exposure.

Gaps in Knowledge

Major gaps exist in our understanding of immune-related toxicity stemming from ST exposure Most immune-based research is focused primarily on effects of the oral cavity, while potential effects of ST use on the systemic immune response is severely lacking. Moreover, our current understanding of immune-related ST toxicity is conflicting, as some evidence suggests that it exerts overt tissue damage through the over-production of immune-mediated cellular machinery, and others note an ST-induced reduction in these same processes. However, it appears clear that ST use may lead to a disruption in the delicate balance of the immune responses involved in inflammation resolution and tumorigenesis. Thus, as disruption of immune homeostasis may result in suppression leading to increased host susceptibility to pathogens and/or cancer, or immune enhancement associated with hypersensitivity and autoimmune responses, more work in this area is needed to better define the immunomodulatory role of ST in general.

Toxicological research into the reproductive effects of ST also falls short The large majority of studies in this area are epidemiologically based and some of these data appear controversial, as the findings are based primarily on two populations, lacking (much) genetic diversity. However, in light of the data (albeit limited) pointing to a relationship between ST use and adverse obstetric outcomes and the serious and long-term consequences associated with such outcomes, more research is needed to better

understand the relationship between ST (in general) and effects on the unborn child.

Toxicological implications of ST on the cardiovascular system are limited and evidence that does exist is conflicting It is currently unclear whether ST use definitively correlates with increased CVD and resulting mortality. Effects of ST on endothelial dysfunction are also being debated. While some human and experimental studies suggest an association between ST and endothelial dysfunction, other investigations fail to support these findings. Increases in blood pressure and heart rate observed in ST users appears to be mediated by elevation in blood nicotine levels, although this hypothesis remains to be proven.

Current ST studies lack product standardization and most human studies fail to consider the specific type of ST being used or their unique components Smokeless tobacco is a general term that represents a large number of different product types each being a unique mixture of different chemicals. Experimental studies are needed that utilize a standardized reference product for each ST type (e.g., pan marsala, gutkha, etc.) similar to the "reference cigarettes" used to define the toxicological effects of cigarette smoke. Controlled tudies such as these will better define the health risks associated with each particular ST product.

Future Research Directions

Adequate delineation of the complexities of the effects of ST will require a broad-based research approach, encompassing clinical data analysis, epidemiological, in vivo, and in vitro methodologies. Approaches should begin with thorough clinical data analysis and epidemiology to provide evidence that could serve as a basis for hypothesis-driven experimental studies of potential adverse health effects. Such an approach could provide the "missing link" between ST use and adverse health outcomes.

There is a critical need for mechanistic studies that relate the effects of ST on these same toxicological outcomes. In vitro cell culture studies and animal models of disease such as atherosclerosis, using apolipoprotein E (ApoE) mice, for instance, may prove to increase understanding of the effect of ST on the cardiovascular system. Moreover, an animal model of diabetes, such as the use of nonobese diabetic (NOD) mice, could increase knowledge of how chronic diseases such as diabetes may be affected by ST use. Considering the rising prevalence and incidence of ST usage, and the potential threat to human health, great advances in our understanding of the toxicological impact of ST use need to be made in the next decade.

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