BILIARY MOTILITY IN HEALTH AND DISEASE

by

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To my parents, for their love and encouragement, and to my wife, who has to put up with my second wife (the IBM up in my study) for the last six months; and for her love, support, tolerance and understanding.

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DECLARATION

I declare that the work contained in this thesis is original and has not previously been submitted for consideration of a Higher Degree or any other professional qualification.

The experimental work for this thesis was carried out while I was employed as a full time Research Fellow in the Gastrointestinal Unit, Western General Hospital, Edinburgh between August 1994 and April 1996. During this period, I was responsible for the organisation of the projects and carried out all the experiments myself.

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ABSTRACT

This thesis examined several aspects of biliary physiology, the incidence of the post-cholecystectomy syndrome and changes in sphincter of Oddi (SO) motility following cholecystectomy.

Gallbladder emptying starts before the stomach begins to empty and before nutrients reach the small bowel to stimulate cholecystokinin (CCK) release. The mechanism of the early phase of gallbladder emptying is unclear. Gallbladder emptying was examined in a group of volunteers after ingestion of a fatty meal, a sham meal and gaseous gastric distension. The early phase of gallbladder emptying was found to be associated with CCK release. In most cases, gaseous gastric distension and sham feeding did not cause significant gallbladder emptying or CCK release but in a minority of individuals early gallbladder emptying followed sham feeding and this was associated with increased plasma CCK concentrations. Early release of CCK could be mediated by a combination of vagal-cholinergic stimulation and neuro-hormonal reflex in response to bombesin release by intragastric nutrients.

The effect of nitric oxide (NO) upon gallbladder motility was examined in a group of volunteers after fatty meal during infusion with NO donors (glyceryltrinitrate and sodium nitroprusside), normal saline and hydralazine as a hypotensive control agent. Postprandial gallbladder emptying was significantly reduced during infusion with the NO donors. This inhibitory effect was independent of hypotension and CCK release. This inhibitory effect of NO donors was also observed on isometric contraction of isolated gallbladder muscle strips.

The effect of NO on the SO was examined by topical infusion of glyceryltrinitrate to the ampulla during SO manometry. Basal tone and phasic activity were both suppressed. This finding may have therapeutic application for stone extraction and duct cannulation during endoscopic retrograde cholangiopancreatography.

Symptoms were assessed in 100 patients before and six months after laparoscopic cholecystectomy (LC) with standard questionnaire. 13% of patients had persistent biliary symptoms. Pre-operative abdominal bloating and consumption of antidepressant were found to be significantly more prevalent in these subjects compared to patients who had successful operations.

SO dysfunction is a cause of post-cholecystectomy pain. We hypothesised that LC could destroy cholecysto-sphincteric nerves leading to SO dysfunction. SO manometry was performed in a group of volunteers before and six months after LC. Following LC, the SO was not inhibited by CCK. This could lead to relative post-prandial biliary obstruction and result in post-cholecystectomy pain in susceptible individuals and to dilatation of the common bile duct.

Several issues and concepts arose from the work of this thesis. The mechanism underlying the early release of CCK needs further investigation. The clinical relevance of the effects of NO upon biliary tract motility remains to be explored. It is hoped that future research in this area will help to clarify these issues.

ABBREVIATIONS

^0C	degree(s) centigrade
CCK	Cholecystokinin
CBD	Common bile duct
EFS	Electrical field stimulation
ERCP	Endoscopic retrograde cholangiopancreatography
FMS	Fatty meal scintigraphy
g	gram
GTN	Glyceryltrinitrate
H	Hydralazine
KCl	Potassium chloride
kg	kilogram
LC	Laparoscopic cholecystectomy
L-NMMA	L-N^G-monomethyl-arginine
MBE	megaBecquerel
MMC	Migrating motor complex
m	milli
M	Molar
mol	mole
μ	micro
n	nano
NANC	Nonadrenergic noncholinergic
NO	Nitric oxide
NOS	Nitric oxide synthase
PC	Post-cholecystectomy
QHBS	Quantitative hepatobiliary scintigraphy
SEM	Standard error mean
SD	Standard deviation
SNP	Sodium nitroprusside
SO	Sphincter of Oddi

PART ONE

INTRODUCTION AND LITERATURE REVIEW

CHAPTER ONE

BILIARY TREE - ANATOMY AND PHYSIOLOGY

1.1 Introduction

Diseases of the gallbladder are common and affect 10 to 20% of the world's population. Biliary motility involves a series of complex interrelationship between gallbladder, cystic duct, common bile duct, sphincter of Oddi (SO) and upper small intestine. Neural and hormonal factors modulate these relationships. Alterations in biliary motility are increasingly implicated in the aetiology of gallstones and post-cholecystectomy pain. The application of modern techniques, such as ultrasonography and SO manometry, to the study of the biliary tract has allowed a better understanding of biliary physiology. This thesis examined aspects of biliary physiology and alterations which occur after cholecystectomy.

1.2 Gallbladder

1.2.1 Anatomy

The gallbladder is a piriform, muscular sac and lies in a depression on the inferior surface of the liver at the junction of the right and left lobes known as the "gallbladder bed". It is divided into four areas: the fundus, the body, the infundibulum and the neck. The *fundus* is the expanded end lying nearest the abdominal wall, usually at the junction of the ninth costal cartilage and the right rectus abdominis muscle. The *body* constitutes the major portion of the gallbladder. It is in direct contact with the first portion of the duodenum and the hepatic flexure of the colon. Inflammation from gallstones can cause a fistula between the gallbladder and the duodenum or between the gallbladder and the colon. It is continuous with the *infundibulum*, a transitional area that leads to the narrow *neck*, which, in turn opens into the cystic duct. Hartman's pouch is a sacculation on the inferior surface of the infundibulum. Gallstones can impact and become trapped in Hartman's pouch, causing obstruction of the cystic duct and acute cholecystitis. Sometimes the inflammatory process can produce obstruction of the common bile duct; the "Mirizzi syndrome".

Histologically, the gallbladder has four layers: mucosa, muscularis, a perimuscularis connective tissue and the serosa. The outermost serosal layer is deficient on the surface of the organ that is in direct contact with the liver. The fibromuscular layer is

composed of fibrous tissues mixed with smooth muscle loosely arranged in longitudinal, circular and oblique bundles. The mucosa consists of a glandular epithelium with columnar cells bearing intestinal like microvilli on their surface and tubuloalveolar glands extending into the lamina propria. It is involved in water and electrolyte absorption.

The gallbladder is innervated by the hepatic branch of the anterior vagal nerve and by the sympathetic nervous system via the coeliac plexus [1]. Fibres carried in the right phrenic nerve may also be distributed to the gallbladder via the hepatic plexus. Autonomic plexuses are found in both the muscular and submucosal layers; and parasympathetic ganglia, predominantly in the body and fundus, have been identified within these plexuses. These intramural plexi receive input from the vagus nerve and the sympathetic nervous system.

1.2.2 Physiology

Gallbladder filling is determined by the rate of bile secretion from the liver and the resistance to flow through the lower end of the bile duct produced by the SO. In humans, the secretory pressure developed by the liver is 39 cm H_2O with a common bile duct pressure of 12 cm H_2O [2]. In the fasting state, the SO is tonicaly contracted to a pressure of 12 to 15 cm H_2O. In addition phasic contractions occur about six times per minute. As the opening pressure of the cystic duct is approximately 8 cm H_2O, the pressure gradient at the SO inhibits flow into the duodenum and favours its entry into the gallbladder.

Gallbladder motility is controlled by endocrine and neural mechanisms. Many peptides function both as hormones and neurotranmitters.

(1) Hormonal factors

CCK is the primary hormone controlling gallbladder contraction. After meals, the release of CCK from the I cell of the duodenal mucosa causes gallbladder contraction and SO relaxation, thereby promoting bile flow into the duodenum [1]. CCK produced a

dose dependent contraction of gallbladder muscle strips in vitro [3]. In healthy subjects, there was a direct relationships between physiological plasma CCK concentrations and gallbladder emptying [4,5]. Maximum gallbladder contraction was obtained 15-30 minutes after exogenous administration of CCK, and 40 to 60 minutes after ingesting a fatty meal [6]. The action of CCK on the gallbladder was mediated by direct binding to a specific receptor which is an 85 to 95-kd protein [7] located on the smooth muscle cells of the gallbladder wall [8]. Blockade of this receptor by a specific CCK-A receptor antagonist, loxigumide, completely prevented CCK-mediated gallbladder contraction in humans [9]. In experiments on cats under anaesthesia with ketamine, Behar and Biancani showed the presence of at least two types of excitatory receptors for CCK in the feline gallbladder; one on postganglionic cholinergic neurones, the other at the gallbladder smooth muscle [10,11]. The effect of CCK on the gallbladder was partially blocked by atropine; it also stimulated the gallbladder smooth muscle directly as the atropine-resistant effect was unaltered by complete denervation with tetrodotoxin [11]. CCK has been identified within vagal neurones of the gallbladder intramural plexus and may act as a parasympathetic postsynaptic neurotransmitter or as a stimulator of acetylcholine release [12,13]. The neuronal and myogenic CCK receptors differ in their sensitivity to CCK. Takahashi et al [14] elegantly demonstrated in an *in vivo* guinea pigs model that the gallbladder response to physiological plasma levels of CCK could be markedly blunted by atropine, whereas supraphysiological concentrations of CCK contracted the gallbladder by an atropine-insensitive pathway. This suggests that at physiological levels, the effect of CCK on the gallbladder is predominantly neural and cholinergic. Therefore only a minor component of the endogenous CCK effect might be myogenic.

The gallbladder does not simply fill passively during periods of fasting and then discharge completely into the duodenum in response to a meal. Partial emptying and refilling occur in association with the migrating motor complexes (MMCs) in the duodenum in humans [2]. The gallbladder periodically empties in relation to phase II of the interdigestive motor activity in the duodenum [15], resulting in emptying of up to

30% of that seen after a meal [16]. This emptying could be abolished by cholinergic blockade with atropine in healthy volunteers [17]. These interdigestive contractions serve to partially empty the gallbladder of viscid bile and this is thought to reduce sludge and gallstone formation. The hormone motilin is not thought to be directly responsible for gallbladder contraction because gallbladder contraction precedes rise in plasma levels of this hormone in humans [17,18].

Other gastrointestinal peptides and neurotransmitters have either cholecystokinetic actions (direct and/or CCK potentiating) or cholecystostatic actions (direct and/or CCK inhibiting). Gastrin belongs to the same family of peptides as CCK. It caused gallbladder muscle contraction in some species, though much less potently than CCK [19] and not at all in man [20]. Secretin, released from the upper gut by acid, had no intrinsic effect on gallbladder muscle contraction although it potentiated CCK effects on the gallbladder [21]. In dogs neurotensin caused gallbladder contraction although its potency was much less than CCK [22]. In man, neurotensin caused gallbladder relaxation *in vivo* but no effect was seen *in vitro* [23]. Pancreatic polypeptide caused relaxation of the gallbladder and decreased intraluminal pressure which encouraged refilling after contraction [24]. The concentration of pancreatic polypeptide remains elevated up to six hours after meals. This suggests that the hormone could have a role in the regulation of gallbladder filling. Vasoactive intestinal polypeptide inhibited the contractile response of the gallbladder *in vivo* [25]. Somatostatin diminished hepatic bile secretion and was also a potent inhibitor of meal or CCK induced gallbladder emptying in man [26].

(2) Neural factors

Neural control of the gallbladder is mediated by intramural plexus that receive input from the vagus, sympathetic nervous system and the intramural plexus of the choledochoduodenal junction. This plexus contains cholinergic, adrenergic, serotonergic and peptidergic neurons [13]. CCK and Vasoactive intestinal peptide have been identified in this plexus [12]. Vagal activity contributes to normal gallbladder tone. Direct cholinergic stimulation by bethanechol produced significant gallbladder emptying and cholinergic blockade with atropine diminished the gallbladder

emptying responses to food and CCK [27,28]. This effect of cholinergic blockade appeared to be mediated by a selective muscarinic M_2 receptor [29]. However, the effect of vagotomy upon gallbladder contraction is unclear. Human and animal studies have shown that truncal vagotomy led to loss of gallbladder tone, gallbladder distension and increased risk of gallstones formation [30]. In contrast, several studies showed increased gallbladder sensitivity to both endogenous and exogenous CCK in vagotomised dogs [31] and humans [32]. The physiologic role of sympathetic innervation is also unclear. Administration of adrenaline, noradrenaline or isoproterenol produced no effect at physiologic doses [33] but adrenergic agents relaxed the CCK stimulated gallbladder [12].

(3) Other hormones and drugs

Women are twice as likely as men to develop gallstones and this may be due to differences in sex hormones. Progesterone inhibits gallbladder contractions. Gallbladder emptying is impaired in pregnancy and during the luteal phase of the menstrual cycle [34]. Apart from effects on gallbladder mucus production, several prostaglandins have been shown to produce gallbladder contraction *in vitro* [35]. Indomethacin, a potent inhibitor of prostaglandin synthesis, relaxed the gallbladder and this might account for its effectiveness as an analgesic for patients with biliary colic [36,37].

Erythromycin, a motilin agonist, produced a dose-dependent emptying of the gallbladder in normal volunteers and in patients with gallstones [38]. Studies on cisapride have produced conflicting results [39,40].

(4) Early gallbladder emptying

A paradox noted by other investigators is that the gallbladder starts to empty almost immediately after ingestion of fatty meal [41,42]. It seems very unlikely that this occurs as a consequence of nutrient induced CCK release from the small bowel since the stomach does not start to empty for several minutes after ingestion. The mechanism for early

gallbladder emptying contraction is unknown but this is discussed further in section 6.4.

1.3 Sphincter of Oddi

1.3.1 Anatomy

The pancreaticobiliary sphincter was first described by Glisson in 1654 [43] but it was named after Ruggero Oddi who characterised it extensively [44]. He was the first person to perform manometric study on the SO. Boyden later distinguished the different parts of the SO in several species and in humans [45].

The right and left hepatic ducts join outside the liver, near the porta hepatis, to form the common hepatic duct. Occasionaly, the right and left hepatic ducts do not join until the cystic duct has joined the right hepatic duct; in that case there is no common hepatic duct. The cystic duct connects the neck of the gallbladder to the common hepatic duct to form the common bile duct. It usually enters the common hepatic duct directly but it sometimes run parallel to or spirals around the common hepatic duct before entering it.

The common bile duct varies in length from 5 to 17 cm. Its diameter varies from 0.3 to 1.5 cm, and can become markedly distended by biliary obstruction. The common bile duct contains scanty smooth muscle arranged in a circular fashion [46]. It has a fibroareolar coat. The lumen is lined by columnar epithelium that is continuous with that of the gallbladder and the other extrahepatic bile ducts. It is divided into four segments: supraduodenal, retroduodenal, pancreatic and intraduodenal [46]. The intraduodenal segment lies posterior and superior to the main pancreatic duct and is about 2 cm long. A number of variations exist in the fusion and entrance into the duodenum of the bile and pancreatic ducts [47]. After running parallel for 0.2 to 1 cm, the two ducts may join outside the duodenum, or they usually form a common channel as they course through the duodenal wall to form the ampulla and terminate at the papilla of Vater; in these cases, a single ostium is identified on the major duodenal papilla during endoscopy. The papilla of Vater is a small, nipple-like protrusion from the pancreatic border of the duodenum, usually at the junction of the second and third

part. The papilla of Vater is rarely larger than 1 cm in diameter from endoscopic study [48]. In about 30% of cases, however, these ducts drain separately into the duodenum (in which case there is no ampulla), or the ampulla is so short (<2 mm) that it is almost non-existent. The bile and pancreatic ducts become narrower as they traverse the papilla of Vater. The transampullary septum, the thin veil of tissue that separates the terminal end of the bile duct, may be an important anatomic structure because it is shared by the two structures as a common wall [49]. The intraduodenal segment of the common bile duct and the ampulla of Vater are surrounded by a smooth muscle sheath. This complex system of circular and longitudinal muscle is called the sphincter of Oddi (SO).

The intrinsic muscle fibres of the SO differ from the musculature of the duodenum both in embryologic development and in histologic features. The SO can be divided into four sections. The sphincter choledochus surrounds the distal common bile duct just prior to joining the pancreatic duct. The sphincter pancreaticus is a circular muscle layer present at the terminus of the pancreatic duct. The muscles of the sphincter choledochus and the sphincter pancreaticus usually interlace in a figure-of-eight fashion. The sheath of smooth muscle surrounding the ampulla is called the sphincter ampullae; it is a continuation of sphincter choledochus. If there is no ampulla, the distal sphincter is called the sphincter of the papilla. The mean extraduodenal length of sphincter is estimated to be 5 mm (1 to 11 mm) and the mean intramural length to be approximately 14 mm (7 to 22 mm). Thus the mean length of the sphincter is around 19mm [50]. However, the physiologic sphincter as based on manometric study, is significantly much shorter with an average length of 10 mm [51].

The human papillary complex passes through the duodenal wall at an acute angle. It is likely that the duodenal wall in this area contributes to the pressure gradient that exists within the papilla of Vater. A contraction of duodenal smooth muscle or a rise in the intraduodenal pressure could increase the transpapillary pressure and thereby prevent the reflux of duodenal contents into the common bile duct and pancreas [52].

Histologically, the SO consists of a scanty layer of longitudinal smooth muscle and a prominent outer layer of circular muscle [53]. Myenteric ganglia are present between these muscle layers and abundant nerve bundles have been found overlying the adventitia [54]. Similar to the gallbladder, the SO is innervated by sympathetic nerves from the coeliac ganglia via the splanchnic nerves which supply motor and inhibitory fibres. Parasympathetic innervation is derived from the vagal nerves which supply motor and sensory fibres. Catecholamines and other gastrointestinal peptides such as galanin, vasoactive intestinal peptides, somatostatin and substance P have all been identified in the neurons [55]. However, the relation between the intrinsic myenteric ganglia and extrinsic innervation remains unknown.

1.3.2 Physiology

The primary function of the SO is to control the delivery of bile and pancreatic juice into the duodenum. The SO also diverts bile into the gallbladder and prevents reflux of duodenal juice into the ducts.

The exact mechanism by which the SO regulates bile flow in response to feeding remains controversial and the physiologic significance of the direction of phasic contractions remains obscure. There is variation between species in the neural and hormonal control of interdigestive and postprandial SO motility.

There is evidence that in herbivorous animals such as the prairie dog [56], American opussum [57], and rabbit [58], the SO exhibits an excitatory response to CCK and feeding. In these animals the SO is believed to actively pump bile into the duodenum. In carnivorous animals such as dogs [59] and man, ingestion of a meal [60] and injection of CCK [61] produces a decrease in SO basal pressure with reduced amplitude and frequency of phasic contractions. This facilitates the flow of bile into the duodenum.

As in the gallbladder, the actions of CCK upon the SO are believed to be mediated by myogenic and neural pathways [11]. CCK stimulated postganglionic nonadrenergic,

noncholinergic inhibitory neurons to relax the SO and this was abolished by tetrodotoxin in anaesthetised cats. CCK also caused SO contraction by stimulating excitatory receptors on the smooth muscle; and this effect became apparent only after complete denervation with tetrodotoxin. This suggests that CCK has a neurally mediated, inhibitory effect that overrides a direct excitatory effect on the SO smooth muscle [11]. This is supported by observation from experimental work on anaesthetised prairie dogs that truncal vagotomy increased baseline resistance to bile flow [62] and increased the frequency and amplitude of SO phasic contractions [63].

The human SO, like the gallbladder, exhibits regular contractile activity during passage of the migrating motor complex of the upper gastrointestinal tract. This was studied by Worthley et al using a manometric catheter through the T-tube of patients undergoing biliary tract surgery [60]. Unlike the duodenum, the SO exhibited regular phasic activity during phase I and contraction frequency increased during phase II of the duodenal cycle. The SO contracted independently of the duodenum. During phase III, the SO contractile frequency was similar to duodenal frequency and its basal pressure increased. These findings suggest that during this phase, bile flow from the bile duct into duodenum is retarded. This is consistent with the finding that gallbladder contraction reached its peak during phase II, just before phase III of the duodenal cycle [15,16]. These interdigestive cyclic contractions may be mediated through the action of motilin.

Phasic contractions of the SO may be antegrade, retrograde or simultaneous. In humans, it appears that phasic contractions have a dual role. Antegrade contractions help to expel small volumes of bile into the duodenum and this prevents reflux of duodenal contents into the biliary tree. The simultaneous and retrograde waves create resistance to flow and the pressure gradient helps to cause influx of bile into the gallbladder, particularly during the interdigestive phase of gallbladder filling. These forceful contractions are thought to help to prevent reflux of duodenal contents into the biliary tree. Feeding causes a decrease in SO motility thereby facilitating the flow of bile into duodenum. It therefore appears that in herbivorous animals, the SO

acts like a pump whereas in carnivorous species like man, the SO acts as a variable resistor in controlling bile flow [64-66].

In both the cat and opossum, there are nerve trunks that connect the gallbladder and SO [67,68]. These nerve trunks connect groups of ganglia found at the junction of cystic duct and bile duct, in the gallbladder wall and the SO. Examination of human gallbladders and SO have confirmed the presence of similar ganglia and nerve plexus [50]. In man, distension of the gallbladder inhibited the amplitude of SO phasic contraction [69]. In cats, this "cholecysto-sphincteric" response could be abolished by interruption of the neural connections between the gallbladder and SO with injection of tetrodotoxin or local anaesthetic agent at the junction between the cystic and common bile duct [70]. This suggests that these connecting nerve trunks could be similar to the CCK responsive inhibitory neurones for the SO. It is not clear whether there is a "pacemaker" controlling SO relaxation.

1.4 Cholecystokinin

1.4.1 Historical

In 1928, Ivy and Goldberg demonstrated that extracts of small intestinal mucosa caused contraction of the gallbladder; they called the active material CCK [71]. In 1943 Harper and Raper extracted a potent stimulant of pancreatic secretion from small bowel mucosa which they named pancreozymin [72]. However, it was not until 1960's that it became feasible to purify and characterise CCK through the meticulous work by Mutt and Jorpes [73,74]. They discovered that CCK was a sulphated tritiscontapeptide amide which possessed gallbladder and pancreatic stimulatory activity and showed that CCK and pancreozymin were a single hormone. Because CCK was discovered first, it became conventional to refer to the polypeptide as CCK. It is now known that CCK has multiple actions. It acts as both a hormone and a neurotransmitter in the brain, the enteric nervous system, and at several gastrointestinal organs.

1.4.2 Structure

CCK is expressed in endocrine cells in the gut and in neurones of the brain and gut [75]. CCK occurs in several bioactive forms and the biogenesis of CCK involves modifications of pro-CCK, the precursor, resulting in extensive molecular heterogeneity. The original material isolated was CCK-33, subsequently CCK-8, CCK-22, CCK-39 and CCK-58 were identified. These are all derived from pro-CCK and are all sulphated on the tyrosine residue at position 7 from the carboxyl-terminal [76]. CCK shares a common five amino acids sequence at the carboxyl-terminus with gastrin (Figure 1.1); the biological activity of CCK is contained in the eight carboxyl-terminus residue, the sulphation on the tyrosine being an absolute requirement. Only 50% of gastrin is in the sulphated tyrosine form which is located at position 6. Desulphation or shifting the tyrosine residue from this position abolishes CCK activity. CCK33 and CCK39 are the major circulating molecular forms constituting up to 55% [77]. Basal plasma CCK concentration, measured by radioimmunoassay, is approximately 2.6 pmol/l and ingestion of a low and medium fat meal induces a rise in plasma CCK concentration to 6.1 pmol/l within 15 minutes [78]; fatty meal is known to increase CCK concentration to approximately 15pmol/l at 60 minute [79]. CCK is almost completely metabolised on first passage through the liver [80] and metabolites are excreted through the kidney [81]. The plasma half life of CCK is about 2.5 minutes in man and the dog [81].

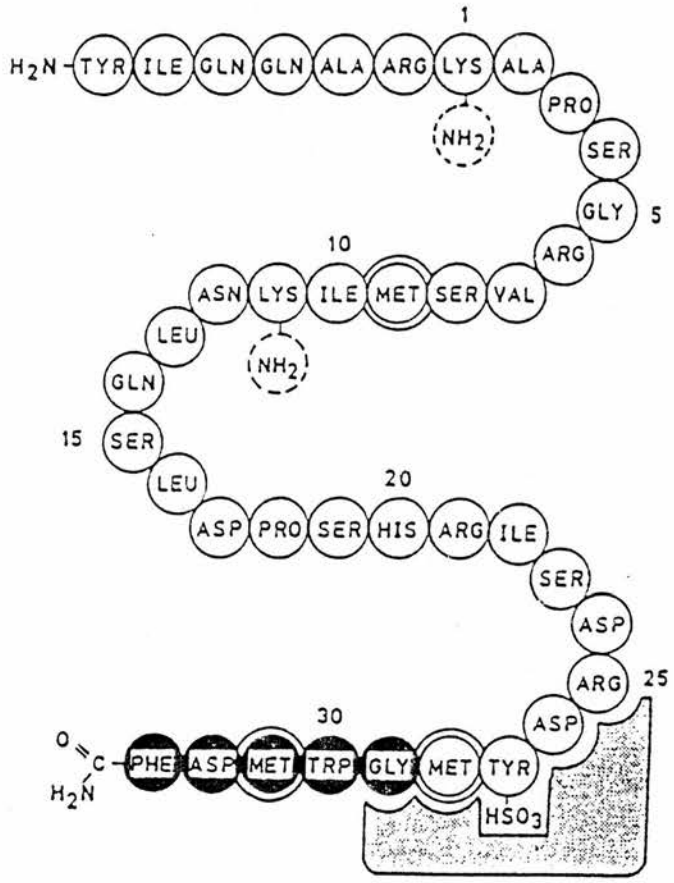


Figure 1.1 Molecular structure of porcine CCK. The filled circles indicate C-terminal pentapeptide sequences (29-33) that is homologous with C-terminus gastrin (Adapted from Rehfeld [82]).

1.4.3 Regulation of cholecystokinin release

(1) Stimulants of cholecystokinin release

CCK is located in the I-cell within the mucosal glands of the proximal small intestine. These cells are most numerous in the duodenum, and become less frequent distally. CCK is also found in the neurones of the central and peripheral nervous system.

In man, plasma CCK concentrations are increased after ingestion of a meal and during infusion of bombesin [83-85]. Fat and protein are the most potent stimuli [83,86]. Intraduodenal infusion of saline does not stimulate CCK release [84]. The role of carbohydrate in stimulating CCK release is rather controversial; Liddle et al [78] demonstrated that carbohydrate could induce release of CCK; several other studies produced conflicting results [83,87]. Triglycerides required hydrolysis and dispersion of fatty acids into micelles to become effective stimulants [88]. Inactivation of lipase activity in the upper small intestine by the lipase specific inhibitor tetrahydrolipostatin inhibited CCK release in response to unhydrolysed fat [88]. In rats, intact protein but not amino acids, stimulated the release of cholecystokinin and pancreatic exocrine secretion [89,90]. In man, protein also required to be digested before it stimulated CCK release [91]. Intraduodenal perfusion of camostate at a concentration that inhibited proteolytic activity, significantly lowered protein-stimulated, but not amino acid stimulated, plasma CCK, gallbladder emptying, and pancreatic enzyme output. That adequate protein digestion and lipolysis is necessary for CCK release, is consistent with observations in patients with exocrine pancreatic insufficiency in whom supplementation of pancreatic enzyme restored impaired postprandial release of CCK and gallbladder motility [92]. CCK release and gallbladder contraction after a fatty meal was impaired in patients with coeliac disease although this could be normalised by ingestion of pre-digested meals [93,94].

Little CCK is released during the cephalic [95,96] or the gastric phase of digestion [87]. CCK was released in anaesthetised dogs by electrically stimulating the vagus nerves

[97], whilst sham feeding [96] and insulin hypoglycaemia as vagal stimulation in man did not consistently lead to CCK release [98]. Vagotomy in man did not influence the release of CCK but increased the sensitivity of gallbladder smooth muscle to this hormone [32]. It therefore appears that only intestinal stimulation results in significant CCK release.

(2) Feedback inhibition

In 1943 Thomas and Crider showed that peptone, soap or hydrochloric acid caused less bile secretion when given in the presence of sodium taurocholate to the canine intestine than when given alone, suggesting that bile had an inhibitory effect on pancreatic secretion [99]. Malagelada and co-workers confirmed this findings three decades later demonstrating in healthy volunteers that bile acids inhibited gallbladder contraction and pancreatic enzyme secretion stimulated by luminal fatty acids and amino acids [100,101]. Cholestyramine, a bile acids sequestrator, markedly increased amino acids stimulated [102] and bombesin-stimulated [84] CCK release and pancreatic protein secretion [102].

In rats, negative feedback regulation of CCK release is mainly mediated by intraluminal protease activity [103,104]. In contrast, considerable controversy exists whether such a regulatory mechanism operates in man. Slaff et al [105] and Owyang et al [106,107] demonstrated in man that exogenous trypsin suppressed CCK release and pancreatic enzyme secretion stimulated by amino acids or fatty acids. However, other studies in man failed to confirm such a feedback mechanism [108-110]. Administration of the protease inhibitor camostate did not affect bombesin stimulated plasma CCK concentration or gallbladder volumes. There could be several explanations for the discordant findings. Firstly, an inhibitor-resistant trypsin may be present in human pancreatic juices and secondly, it may be necessary to inhibit all three pancreatic endopeptidases because all of them have inhibitory effect on CCK release. Thirdly, it may be necessary to provide appropriate amino acids or fatty acids as stimulus before protease inhibition can be demonstrated. One recent study using a sensitive and specific CCK radioimmunoassay showed that intraduodenal

camostate did not augment CCK released by intraduodenal amino acids, albumin [91] and infusion of bombesin [84].

Dietary stimulation appears to be the most important factor regulating CCK release under physiological conditions. The intensity of CCK release is controlled by CCK expressing genes; intraduodenal administration of trypsin inhibitor significantly increased CCK mRNA. In contrast, fasting was associated with a decrease of duodenal CCK mRNA levels and this was restored rapidly by refeeding [111]. This appeared to be a specific effect upon CCK genes as fasting and refeeding did not produce any changes in either duodenal somatostatin or its mRNA concentration.

Other gut hormones such as somatostatin and bombesin, which are also modified by diet, also affect CCK release. There is some evidence that PYY, an enterohormone released from the distal small intestine and colon, may inhibit the release of CCK [112].

1.4.4 Cholecystokinin receptors

CCK exerts its effects through G protein-coupled seven transmembrane domain receptors coupled to stimulation of phospholipase C [113]. There are two receptors subtypes. CCK-A receptors, characterised most extensively on pancreatic acinar cells and named for their alimentary origin, and CCK-B(brain) receptors. Both forms have now been described in both the gut and brain. CCK-A receptors have 1000-fold higher affinity for sulphated CCK than for gastrin, whether sulphated or not [114]. CCK-B receptor is similar or identical to the peripheral gastrin receptor. CCK shows different contractile responses in stomach, intestine and gallbladder which could be attributed to the different cellular location of CCK receptors. In the intestine, the response is largely mediated by neural CCK receptors and depends also on release of acetylcholine from cholinergic neurons. In the fundus of the stomach and gallbladder, the response is largely mediated by smooth muscle receptors [115].

1.4.5 Regulation of gastric acid secretion and motility by cholecystokinin

CCK has been implicated in the control of gastric acid secretion. The effects of CCK on gastric acid secretion are rather complex and were poorly understood until recently. Exogenous CCK or its analogue, caerulein, administered alone in fasted human caused weak stimulation of gastric acid secretion but inhibited pentagastrin-induced acid secretion [116]. The parietal and histamine-producing cells express a CCK-B/gastrin receptor that mediates gastrin-induced gastric acid secretion. The structural similarity between CCK and gastrin and their ability to bind equipotently to this receptor as agonists suggests that both hormones can stimulate gastric acid secretion [117]. The inhibitory effect on acid secretion is postulated to be mediated through the CCK-A receptor located on somatostatin-producing cells [118]. Loxiglumide, a specific CCK-A receptor antagonist, increased both gastric acid secretion and plasma gastrin in response to a peptone meal and ordinary meal in a group of healthy volunteers[119,120]. Loxiglumide also further enhanced acid secretion induced by caerulein/CCK-8 and gastrin releasing peptide [119,120]. However, loxiglumide failed to affect basal gastric acid outputs and plasma gastrin concentrations [119,121]. The findings from these studies suggest that CCK does not play any role in the control of basal gastric secretion but post-prandial CCK release has a tonic inhibitory influence upon both gastric acid output and gastrin release via the CCK-A receptor located on somatostatin releasing D cells [118]. It is postulated that CCK stimulates the paracrine secretion of somatostatin which inhibits acid output from parietal cells and gastrin released from G-cells, and this inhibition can override a direct stimulation of acid secretion via CCK-B/gastrin receptors on parietal cells.

In addition to its effect on gastric acid secretion, CCK also has regulatory effects on gastric motility. Meyer et al showed that loxiglumide reduced colonic transit time and gastric emptying of liquid meal in healthy volunteers [122]. Fried et al later reconfirmed that physiological concentration of CCK inhibited gastric emptying [123]. It is possible that CCK principally controls liquid emptying since the CCK antagonist L364,718 had no effect upon gastric emptying of solid-liquid meal [124]. Physiological doses of CCK infusion were also known to stimulate pancreatic

secretion (see 1.4.7). Therefore CCK acts as a common regulator for gastric emptying and pancreatic secretions so as to optimise the ratio of nutrients entering the upper small intestine to pancreatic digestive juices. Little is known about the mechanisms by which CCK regulates gastric motility. It may act directly through specific receptors on smooth muscle cells or through the release of other substances. As eluded to earlier, CCK is a potent releaser of somatostatin from antral mucosa [118], a peptide also known for its inhibitory actions on gastrointestinal motility. It may also act as a neurotransmitter as the inhibitory effect of CCK on gastric emptying could be prevented by destroying capsaicin-sensitive vagal afferents in rats [125].

1.4.6 Regulation of pancreatic secretion and growth by cholecystokinin

(1) Secretion

In contrast to the complete blockade of CCK-induced gallbladder contraction, loxiglumide only reduced CCK-induced postprandial pancreatic secretion by 50% [126]. The inability of the CCK antagonists to completely inhibit postprandial exocrine pancreatic secretion of enzymes suggests that other hormones and neural mechanisms are also involved in the regulation of meal-stimulated pancreatic secretion. CCK was found to be a strong stimulant of pancreatic enzyme secretion but was probably not the major regulator of water and bicarbonate secretion [127]. It produced marked augmentation of bicarbonate secretion in man when combined with secretin [128].

Recent data suggests that CCK stimulates pancreatic secretion not only through specific receptors at the acinar cells but also through interaction with the cholinergic system. *In vitro* studies with dispersed pancreatic acini showed that CCK-stimulated amylase release was insensitive to atropine or tedrodotoxin, indicating direct action on pancreatic acini [129]. *In vivo* studies in man showed that pancreatic secretion stimulated by CCK could be blocked by atropine, suggesting action via a cholinergic pathways [130,131]. Atropine and loxiglumide both inhibited pancreatic secretion but inhibition by atropine was more pronounced at physiological than at supraphysiological CCK doses [131]. This is consistent with observations reported by

Malagelada that the secretory response induced by small but not high doses of CCK was reduced by vagotomy [132]. Using an *in vivo* rat model, Owyang et al demonstrated that physiological doses of CCK stimulated the vagal afferent pathway [133]. Therefore the action of CCK on pancreatic secretion is modulated by interactions of its action directly on acinar cells and cholinergic neurones.

2) Pancreatic growth

In the rat repeated injection of CCK analogues caused marked increases in pancreatic weight due to hypertrophy and hyperplasia [134]. It is also known that feeding of trypsin inhibitors and pancreaticobiliary diversion stimulated pancreatic growth [135,136]. Such inhibition or diversion of proteolytic enzymes increased plasma concentration of CCK. The role of CCK as a cancer growth promoter is less clear. CCK was shown to have trophic effects upon cancer cell lines derived from pancreatic exocrine glands or ducts [137]. Despite the marked hypertrophy and hyperplasia induced by CCK, no neoplastic nodule was observed in rats exposed to trypsin inhibitors [137].

1.4.7 Regulation of intestinal motility

Ingestion of a fatty meal lowered the basal lower oesophageal sphincter pressure. Although the mechanism for this observation remains unclear, CCK released by fatty meal could be responsible [138]. Infusion of CCK induced a dose-dependent reduction in lower oesophageal sphincter pressure [139,140] but this reduction in pressure has only been consistently shown with supraphysiological doses of CCK [141,142]. The role of CCK-induced lower oesophageal sphincter relaxation in gastro-oesophageal reflux after a fatty meal in healthy subjects is therefore probably of minor importance.

CCK analogues increased transit of contrast material through the human small intestine [143]. CCK increased the frequency of electrical spike potential in human colon *in vitro* [144]. The colonic electrical pattern typical of the gastrocolonic response to a fat meal could be reproduced by intravenous CCK.

1.4.8 Regulation of blood flow

CCK caused vasodilation in upper small intestine, and increased blood flow in the liver of dogs [144] and pancreatic microcirculation [145]. Whether the CCK induced increases in capillary blood flow is a direct effect of CCK or is a result of a paracrine operating system remains to be elucidated.

1.4.9 Effects of cholecystokinin on appetite

High concentrations of CCK are present in the cerebral cortex and limbic system and is colocalised with dopamine in some neurones [146]. CCK has been shown to affect various responses in central nervous system including satiety and anxiety.

CCK has been proposed as a major mediator of the satiety response that leads to cessation of feeding when food is placed in the stomach or intestine. Physiological, post-prandial CCK concentrations significantly increased satiety and decreased food intake in humans [147]. Intraduodenal infusion of fat decreased food intake when compared with saline infusion; this effect could be prevented by loxiglumide [148]. The mechanisms underlying the satiety effects of CCK are unclear; there could be paracrine or neurotransmitter effects. Subdiaphragmatic vagotomy blocked this effect, suggesting that it was peripherally mediated [149].

1.4.10 Effects of cholecystokinin on anxiety

Intravenous injection of CCK-4 provoked panic-like attacks in normal subjects and this anxiogenic effects was most likely to be mediated centrally [150]. The receptor responsible is probably the CCK-B receptor because activation of this receptor caused anxiogenic-like effect in rats, whereas receptor blockade alleviated anxiety [151]. The CCK-B receptor is present throughout the brain and is similar to the peripheral gastrin receptor. It is interesting to note that pentagastrin injection could provoke panic attacks [151].

1.4.11 Measurement of cholecystokinin

(1) Radioimmunoassay

Many problems in the developments of a sensitive and specific radioimmunoassay for CCK could be related to the structure of the peptide, which displays remarkable similarities to gastrin in their active moeities; sharing an identical amidated pentapeptide at the carboxyl end (figure 1.1). Since the carboxyl-terminal pentapeptide is highly immunogenic, this similarity caused considerable problems in raising specific antisera directed towards biologically active molecular forms of CCK as antibodies raised towards this region often had cross reactivity with gastrin. On the other hand, antibodies directed towards the mid portion or amino-terminal antigenic determinant may be species specific and may fail to measure small CCK fragments such as CCK-8 that contains the biologically active carboxyl-terminal [82]. A sensitive and specific assay should measure sulphated CCK-8 and larger sulphated CCKs with equimolar potency without reacting with gastrin. As shown in figure 1.1, such an assay should be specific for the amino-terminal part of CCK-8 which contains the sulphated tyrosine residue. Recently, antibodies have been raised that have high specificity for this biologically active sulphated C-terminal region of CCK [75,82]. A further difficulty related to preparation of a tracer which recognised antibodies directed against the biologically active part of the CCK sequence. It was difficult to perform oxidative iodination of this tracer without damaging the biological activity. This was overcome by non-oxidative coupling of ^{125}I-hydroxyphenylpropionic acid-succinimide ester by a modification of the Bolton-Hunter procedure to the CCK-33 and latterly, CCK-8.

Heterogeneity is a particular difficulty for CCK since CCK antibodies may possess different affinities for the various molecular forms of CCK circulating in plasma (CCK-8, CCK-33, CCK-39 and CCK-58). This may introduce underestimation or overestimation of CCK [75].

Plasma CCK concentrations measured in the circulation by sensitive and specific radioimmunoassays average 1 pM in the fasting state and 5-10 pM after fatty meals [78,152].

(2) Bioassay

The basic unit for biological measurement of CCK is the Ivy Dog Unit, defined as the amount of material that produces optimal contraction of the canine gallbladder after rapid intravenous injection. The standard method for bioassay of natural CCK is contraction of *in situ* guinea pig gallbladder. *In vitro* bioassays have been developed for estimation of CCK activity in human serum. Marshall et al developed a method of using rabbit gallbladder that were first exposed to normal serum and CCK-like activity was measured by contractility [153]. There are noncholecystokinin peptides that cause gallbladder contraction; therefore bioassay of this type tends to over estimate CCK concentrations.

Liddle et al developed a novel method of CCK bioassay by utilising amylase released from pancreatic acini [78]. Plasma is extracted, concentrated and incubated with acini. Gastrin does not interfere significantly with the assay; no other CCK-like bioactivity has so far been identified.

CHAPTER TWO

NITRIC OXIDE AND THE BILIARY SYSTEM

2.1 Discovery of nitric oxide

The striking inability of blood vessels to respond to vasodilating substances in the absence of endothelium was resolved by the discovery of an "endothelium-derived relaxing factor" that subsequently was shown to be identical to nitric oxide (NO) in 1987 [154,155]. NO is the molecule responsible for the stimulation of guanylate cyclase in a number of tissues including the brain and is also a cytotoxic factor released by activated macrophages. NO is synthesized from the amino acid, L-arginine, by a family of enzymes named NO synthase. The L-arginine : NO pathway has also been identified in many other tissues, including platelets, adrenal glands, hepatocytes, Kupffer cells, the lung and several cell lines. NO has a half-life of less than 10 seconds because it is rapidly oxidised to inorganic nitrite and nitrate. NO is also destroyed by superoxide anion. Superoxide dismutase protects NO from breakdown by superoxide anion [156,157]. NO binds to oxyhaemoglobin and other haem-containing proteins; its biologic actions are rapidly terminated by binding to oxyhaemoglobin [158,159].

2.2 Actions of nitric oxide

2.2.1 Cardiovascular system

Vascular endothelial cells synthesize NO enzymatically from the terminal guanidine nitrogen atom of L-arginine by the enzyme nitric oxide synthase (NOS), with L-citrulline as the coproduct [160]. The enzyme is constitutive, calcium- and calmodulin dependent, and releases picomoles of nitric oxide in response to stimulation [161]. This reaction is specific, as a number of analogues of L-arginine, including its D enantiomer, are not substrates. One analogue, the methylated L-arginine analogue N^G-monomethyl-L-arginine (L-NMMA), inhibits this synthesis in a dose-dependent and enantiometrically specific manner. NO formed by endothelium diffuses to nearby smooth muscle cells, in which it binds to ferrous iron in the haem prosthetic group of the soluble guanylate cyclase (sGC), resulting in enhanced synthesis of cyclic GMP from guanosine triphosphate (GTP). This increase in cyclic GMP in the smooth muscle cells lead to their relaxation [162].

L-NMMA induced direct vasoconstriction in the brachial artery and attenuated vasodilatation induced by bradykinin and acetylcholine [163], whereas it had no such effect on the unstimulated hand veins. This suggests the arterial side of the circulation has a continuous release of NO that maintains vasodilator tone. L-NMMA constricted vascular beds and produced a hypertensive response in healthy humans [164]. The vaso-constrictor properties of L-NMMA is entirely endothelium-dependent and results from the inhibition of the endogenous vasodilator mechanism, the L-arginine : nitric oxide pathway. In rings of atherosclerotic arteries as compared with rings of normal arteries, the endothelium-dependent relaxation was decreased and the responses to vasoconstrictors were often enhanced [165].

Platelets generate NO which inhibits platelet aggregation and adhesion by a mechanism dependent on cyclic GMP [166]. Aggregation induced by collagen is accompanied by an increase in intraplatelet levels of cyclic GMP but not adenosine-3',5'-cyclic monophosphate (cAMP). L-NMMA inhibits this increase in cGMP and enhances aggregation.

A calcium-independent isoform of NOS can be induced in vessel walls by cytokines and by endotoxin lipopolysaccharides, which act through the release of cytokines. This induction occurs in endothelial and smooth muscle cells of vessels and the myocardium. This leads to vascular relaxation that is resistant to vasoconstrictors but can be prevented by NOS inhibitors and glucocorticoids. NO released by this inducible NOS accounts for the vasodilation and hypotension characteristics of septic shock as well as the hypotension induced by cytokine therapy in patients with cancer [61]. In patients with septic shock, low doses of N^G-monomethyl-L-arginine has been shown to reverse the hypotension associated with septic shock [167].

2.2.2 Respiratory system

Inhaled NO selectively dilates pulmonary vasculature and may protect against adult respiratory distress syndrome [168]. It may be useful in patients with pulmonary hypertension which has been found to be associated with diminished expression of endothelial NOS [169]. It is postulated that decreased expression of NOS may contribute to the excessive growth of the tunica media observed in this disease.

2.2.3 Immunological system

The activation of macrophages by lipopolysaccharide and interferon gamma, results in induction of the calcium-independent NOS. This induction, which is inhibited by glucocorticoids, results in the sustained production of NO, which diffuses to target cells such as tumour cells, bacteria, fungi, and helminths [170]. In these cells NO combines with iron-sulphur centres in key enzymes of mitochondrial respiratory cycle and pathway for DNA synthesis. These enzymes are subsequently inhibited and result in cell death [171].

Increasing evidence indicates that NO may play a part in acute and chronic inflammation. Nitrite concentrations in plasma and synovial fluid were increased in patients with rheumatoid arthritis and osteoarthritis [172]. Treatment with inhibitors of NOS reduced the degree of inflammation in rats with acute inflammation [173] or adjuvant arthritis [174], whereas L-arginine enhanced it. Increased urinary nitrate excretion was observed in human subjects with diarrhoea and fever [175]. The colonic synthesis of nitric oxide was increased in patients with ulcerative colitis [176] and inhibitors of NOS ameliorated experimentally induced chronic ileitis [177].

The origin of NO in the inflammatory process is unclear, but it could come from blood vessels, neutrophils and macrophages. NO may interact with oxygen-derived radicals to generate molecules that could enhance its cytotoxicity [163]. There are reports suggesting that inhibitors of NOS [164,178] and NO donors [179] protect against some forms of injury. This is probably due to the dual nature of NO, which is cytotoxic and a vasodilator with potentially protective properties. At present, the biological significance of NO production by inflammatory cells remains unclear but

is likely to be involved in vasodilation, formation of oedema, modulation of sensory nerve endings and tissue cytotoxicity. The release of NO may be a mechanism for controlling the levels of O_2^-. NO and O_2^- are known to interact rapidly to form the peroxynitrite anion, which decomposes once protonated into potent oxidants OH and NO_2 [180]. OH and NO_2 oxidises sulphydryl groups and react with metal ions [181]. It has been shown that exposure of colonic mucosa to peroxynitrite results in significant tissue injury [182]. It has been suggested that the protective action of superoxide dismutase in ischaemic tissue may be due in part to the prevention of the formation of peroxynitrite and hence of these highly toxic radicals. However, at present, there is no evidence for the formation of the peroxynitrite radical or its decomposition into OH and NO_2 *in vivo,* and therefore it remains to be established whether the interaction between NO and O_2^- leads to an increased toxicity or is a neutralizing mechanism for two toxic radicals.

2.2.4 Nervous system

(1) Central nervous system. Neurotransmission by agents such as acetylcholine, glutamate and glycine has long been known to be associated with elevated cyclic GMP levels in the brain and particularly in the cerebellum [183]. In 1982, the endogenous activator of the soluble guanylate cyclase in the brain was identified as L-arginine [184]. Addition of L-arginine to rat synaptosomal cytosol in the presence of NADPH as a cofactor, resulted in the formation of NO and citrulline and was accompanied by stimulation of soluble guanylate cyclase [185]. Both of these processes were inhibited by haemoglobin and L-NMMA. These data shows that the rat brain possesses NOS which is Ca^{2+} dependent.

Cytosolic preparations of different brain regions showed that the highest concentration of NOS was present in the cerebellum, followed by the hypothalamus and midbrain, striatum, and hippocampus, with the lowest activity found in the medulla oblongata [186]. Histochemical studies using antibodies to NOS have shown it to occur widely in the central nervous system, primarily in neurons and also in vascular endothelium, with no glial localisation [187]. The biological function of

L-arginine : NO in the brain remains to be elucidated. It is possible that NO is involved in the short term effects of excitatory amino acids as well as in their long-term effects on brain development, learning and memory [188]. It may also play a role in the pathology of the central nervous system. Localisation of NOS corresponds closely with that of NADPH diaphorase in both the brain and the peripheral nervous system [189]. NADPH neurons are selectively resistant to degeneration in conditions such as stroke, Huntington's chorea, Alzheimer's disease and in animals with cerebral ischaemia. Thus, neurones that release NO may be resistant to its cytotoxic actions. Microglial cells, which are of monocyte-macrophage lineage can express the inducible form of NOS [190]. These cells have been implicated in the pathogenesis of diseases such as multiple sclerosis, Alzheimer's disease, Parkinson's disease and the dementia of Acquired Immune Deficiency Syndrome [191].

(2) Peripheral nervous system.

NO may contribute to sensory transmission and may also be the transmitter or modulator in nonadrenergic noncholinergic (NANC) nerves in the gastrointestinal tract (see 2.2.6). It is also responsible for the relaxation of the corpus cavernosum and thus penile erection in human [192]. Immunohistochemical evidence of nerves containing NO has been found in human penile tissue [193]. NO also contributes to the NANC vasodilatation and relaxation of human tracheal muscle [194]. There is therefore a widespread system of nerves throughout the body that use NO as a neurotransmitter and there seems to be NO-dependent dilator tone that is crucial to the physiologic function of these organs.

2.2.5 Nitric oxide and the endocrine system

NADPH and Ca^{2+}-dependent NOS has been identified and characterised in both cortex and medulla of the adrenal gland [195]. The functional importance of this L-arginine : NO pathway in regulating adrenal cortex and medulla function is not clear. cGMP has been implicated in both catecholamine secretion [196] and

steroidogenesis [197]. Rat mast cells have also been shown to produce an NO-like substance that modulated the release of histamine [198].

2.2.6 Gastrointestinal systems

Present evidence indicates that NO mediates relaxation of the muscularis externa and may play an important role in gastrointestinal mucosal blood flow, mucosal protection, haemodynamic responses to liver disease, regulation of hepatocyte function and mediation of hepatoxicity.

(1) Nitric oxide and neural inhibition of gastrointestinal motility.

The identity of the nonadrenergic noncholinergic (NANC) inhibitory neurotransmitter that mediates smooth muscle relaxation remained unclear till recently. Adenosine triphosphate and vasoactive intestinal peptide have been proposed as the neurotransmitter but in many gastrointestinal smooth muscle preparations, neither substance appears to be involved [199,200]. In the early 1980's, studies by Gillespie et al, demonstrated that a nonpurinergic, nonpeptidergic neurotransmitter was involved in NANC neurotransmission in rodent and bovine genitalia-associated smooth muscles. It was subsequently shown that NO mimicked NANC relaxation, blockers of NO synthesis inhibited this effect and L-arginine reversed this inhibition in the rat anococcygeus muscle [201,202]. Bult et al provided evidence that NO was released on stimulation of NANC nerves in the canine ileocolonic junction [203].

NANC neurotransmission has been extensively studied in the smooth muscle from the lower oesophageal sphincter, gastric fundus, gallbladder, SO, ileocolonic junction and internal anal sphincter.

(2) Lower oesophageal junction.

The lower oesophageal sphincter provides a physiological barrier that prevents reflux of gastric contents into the oesophagus. Stimulation of NANC nerves produced relaxation of the lower oesophageal sphincter and contraction of circular smooth

muscle [204]. Electrical stimulation produced a frequency dependent relaxation of lower oesophageal sphincter that was accompanied by an increase in cyclic GMP in human (maximum relaxation at 8.0 Hz with a range of 1Hz to 10 Hz, 1.0 msec, 65v) [205] and opussum (10Hz, 1.0 msec, 40 v) [206]. The electrically induced relaxation could be abolished by tetrodotoxin but was unaffected by atropine and guanithidine indicating the relaxation responses are NANC in origin. Furthermore, the NANC induced relaxation was inhibited by the NOS inhibitor, L-*N*-nitroarginine (L-NOARG) [207]. NOS activity, determined by the transformation of ^{14}C-L-arginine into ^{14}C-citrulline, was detected in healthy human oesophageal tissue homogenates but reduced in patients with achalasia [208]. However, NOS activity could also be detected in tissue homogenate of smooth muscle and striated muscle segments [209]. Immunohistochemical staining of human oesophageal tissue with polyclonal antibody raised against a peptide sequence of rat brain NOS confirmed the presence of NOS in the myenteric plexus of the gastro-oesophageal junction of healthy control but reduced in patients with achalasia [208] and congenital oesophageal stenosis [210]. NO was released upon stimulation (10Hz, 1.0 msec, 30 v) of intrinsic nerves of the lower oesophageal sphincter as detected by a chemiluminescence NO analyser [211]. The NOS identified required NADPH, Ca^{2+}, calmodulin activity and could be inhibited by L-NNA [209]. Injection of aqueous solution of NO gas has been reported to relax gastro-oesophageal sphincter in patients with achalasia [212].

(3) Gastric smooth muscle

The fundus of the stomach actively dilated to accomodate the intake of food with little increase in intragastric pressure [213]. This relaxation occurred predominantly in the proximal stomach and was more pronounced after a liquid meal. This response was neurally mediated but the nerves involved were neither adrenergic nor cholinergic. Desai et al [214] demonstrated that this adaptive relaxation was mediated by NANC and the neurotransmitter was indistinguishable from NO. Incubation of guinea-pig stomach with tetrodotoxin abolished the response which was not abolished by incubation with guanethidine and atropine. Incubation with L-NMMA abolished this adaptive relaxation. L-arginine reduced the amplitude of spontaneous

contraction of canine antral smooth muscle *in vitro* and sodium nitroprusside, a NO donor, hyperpolarised resting membrane potential and decreased contractile responses to acetylcholine [215]. In contrast, L-NMMA augmented acetylcholine induced contraction. NOS has been identified through immunohistochemistry techniques in the myenteric neurones of gastrointestinal tract [187]. Konturek et al demonstrated that sublingual glyceryl trinitrate (a NO donor) inhibited gastric emptying and antral motility after liquid meals in healthy volunteers [216]. The involvement of NO in the regulation of gastric emptying in human was subsequently confirmed by the same investigators with the infusion of L-NMMA. L-NMMA caused a significant reduction of the gastric emptying half-time which could be completely reversed by addition of L-arginine to the L-NMMA infusion [217]. The increase of gastric emptying half time was thought to be due to the suppression of receptive relaxation of the proximal stomach.

(4) Gastric mucosal blood flow and secretion

Both local administration of acetylcholine and vagal stimulation induced vasodilatation of gastric mucosa [218]. There is evidence implicating endogenous NO in modulating the resting gastric microcirculation. Studies using hydrogen gas clearance [219], radiolabelled microspheres technique [220] and laser Doppler flowmetry [221] have demonstrated that intravenous administration of L-NMMA reduced resting gastric mucosal blood flow. Intra-arterial infusion of glyceryltrinitrate induced vasodilatation in the mucosal microcirculation [222]. The mucosal hyperaemia in rats induced by intravenous pentagastrin was attenuated by pre-treatment with low dose L-NMMA[222]. However, L-NMMA had no effect upon peak pentagastrin-induced acid secretion, indicating an effect on the microcirculation independent of secretory modulation.

NO may play an important role in gastric mucosal protection. Topical application of either nitrosothiol *S*-nitroso-*N*-acetyl-penicillamine (SNAP), which spontaneously liberates NO or isosorbide dinitrate to the rat gastric mucosa *in vivo* augmented the thickness of the overlying mucus gel [223]. NO may interact with other sensory

neuropeptides in the regulation of gastric mucosal integrity. Administration of L-NMMA induced gastric mucosal injury in rats pre-treated with indomethacin and two weeks of neurotoxic dose of capsaicin to deplete sensory neuropeptides [224]. The stimulation of afferent neurons by lower dose of intragastric capsaicin protected the gastric mucosa against damage induced by ethanol [225,226] and acidified aspirin [227]. This protection appeared to depend on the marked vasodilation elicited by capsaicin in the gastric mucosal microcirculation. Capsaicin sensitive neurons contain a number of vasoactive peptides, including substance P, VIP and calcitonin gene-related peptide (CGRP). CGRP appears to be the major peptide released from capsaicin sensitive neurons that is involved in mucosal vasodilation as CGRP receptor antagonist ($CGRP_{8-37}$) inhibited the hyperaemic response to capsaicin [228]. In ethanol-induced mucosal damage models in rats, $CGRP_{8-37}$ dose dependently attenuated the protective effect of CGRP and this effect could be reversed by L-arginine. Protection by CGRP was not associated with increased prostaglandin formation [229]. These findings suggest that CGRP is an essential mediator of the protection elicited by stimulation of capsaicin-sensitive neurons and the protective effects of CGRP depends on the L-arginine : NO pathways.

Apart from stimulation of capsaicin sensitive neurons, pentagastrin has also been reported to protect against ethanol-induced mucosal damage in rats [230]. These effects have demonstrated to involve capsaicin afferent neurons, CGRP and the L-arginine:nitric oxide pathway [231]. NOS activity was shown to be increased in enterochromaffin-like cells associated with hypergastrinaemia produced by omeprazole in rats [232].

NO can be formed intragastrically from nitrite and nitrate in swallowed saliva and from nitrate rich containing food such as lettuce. Intragastric production is probably non-enzymatic, and requires an acidic environment as the amount of NO could be reduced by pre-treatment with proton pump inhibitor in a group of healthy volunteers [233]. Patients with duodenal ulcers had higher gastric antral and fundic inducible NOS activity than normal subjects [234]. NOS activity in *Helicobacter pylori* positive

subjects with duodenal ulcers was two fold higher than *Helicobacter pylori* positive normal subjects. The enhanced inducible NOS activity is probably derived from inflammatory neutrophils and macrophages. NO combined with superoxide yielding peroxynitrite which subsequently decomposed to highly toxic radicals OH and NO_2 that might induce tissue injury [180]. NO might also delay proximal duodenal contractions thus increasing the exposure of duodenal mucosa to gastric acid.

(5) Intestinal smooth muscle

NOS has been localised in the myenteric and deep muscular plexuses [187,235,236] and NO participated in the regulation of small intestine myoelectric activity both in mammalian and nonmammalian species [237]. Infusion of NOS inhibitors transformed the postprandial motility into a fasting motor pattern. Moreover, the infusion of a NO donor disrupted the MMC and generated an irregular spiking activity similar to that observed during the postprandial state [237]. NO induced NANC relaxation in circular muscle strips of the canine terminal ileum, ileocolonic junction [238,239] and colon [240]. Incubation with NOS inhibitor increased basal tension of the circular muscle, which could be partly reversed by L-arginine. Intravenous injection of lipopolysaccharide increased the constitutive and inducible forms of NOS in the small intestine and accelerated gastrointestinal transit time. This rapid intestinal transit time could be reversed by intravenous L-NMMA [241].

The internal anal sphincter is innervated by excitatory adrenergic innervation and inhibitory NANC. The NANC nerves relaxed the IAS in response to rectal distension, the recto-anal reflex [242,243]. VIP has been proposed as a mediator of NANC relaxations in this sphincter [243]. However, none of the antagonists to VIP studied so far is capable of completely blocking the effect of NANC stimulation, implying that another NANC transmitter is involved. L-NNA reduced relaxation induced by transmural field stimulation in isolated muscle strips of the opussum but the inhibitory response to VIP was not affected by pre-treatment with L-NNA [244]. Electrical field stimulation (EFS) relaxed isolated strips of human internal anal sphincters in the presence of atropine and guanethidine [245,246]. Sodium nitroprusside,

a NO donor, mimicked relaxation produced by EFS (frequency 10Hz, 10V, 0.5ms duration) and this relaxation could be inhibited by NOS antagonists and oxyhaemoglobin, a scavenger of NO.

NO induces vasodilation in the rat isolated mesenteric vascular bed [247] and L-NMMA increased tone and inhibited the vasodilation produced by acetylcholine in human isolated omental arteries [248]. Using a radiolabelled microspheres technique, L-NNA has been shown to decrease blood flow in the duodenum, jejunum, caecum and colon [249].

Thus the evidence accumulated to date support strongly an involvement of NO in NANC inhibitory neurotransmission. Conventional neurotransmitters such as acetylcholine and noradrenaline are stored in membrane bound vesicles before their release from presynaptic nerves, and they influence postsynaptic cells by interacting with membrane bound receptors on the cell surface. NO is clearly not bound in vesicles and challenges classic concepts of neurotransmission. Firstly, its receptor, guanylate cyclase, is cytosolic rather than membrane bound. Secondly, NO is both labile and very lipid soluble. It is thus unlikely to be packaged in membrane bound vesicles before release. It has been suggested that NO is stored in a more stable form, bound to another molecule such as cysteine, and there is some evidence supporting this [240]. Alternatively, NO can be produced on demand, and it is conceivable that depolarisation makes calcium available for NOS activation within the presynaptic neuron. The mechanism by which NO causes smooth muscle relaxation has not been established. It is recognised that, by activating soluble guanylate cyclase, NO increases synthesis of cGMP and this has been shown to produce membrane hyperpolarisation in colonic smooth muscle [250]. Such membrane changes are the hallmark of NANC nerve mediated relaxation and are thought to result from an increase in potassium conductance [240]. The processes responsible for electromechanical coupling include enhanced Ca^{2+} sequestration and reduced sensitivity of the contractile apparatus to Ca^{2+}.

The high reactivity of NO with a variety of biologically relevant molecules and its short half life under physiologic conditions have led several investigators to postulate that NO-like activity may be stabilised in vivo [251-254]. In particular, S-nitrosothiols (RS-NO) formed from the nitrosation of SH groups of low molecular weight thiols and proteins, are a class of relatively stable NO adducts that form under physiologic conditions and retain smooth muscle inhibitory activity in gastrointestinal and non-gastrointestinal tissue.

(6) Liver and pancreas

In the pancreas, L-NNA decreased canine pancreatic blood flow under resting conditions and following stimulation with secretin and CCK and this led to decreased pancreatic secretion [255].

Rat Kuppfer cells cocultured with hepatocytes and stimulated with lipopolysacharide induced a significant suppression of hepatocytes total protein synthesis, but only when L-arginine was present in the medium [256]. This effect was associated with formation of nitrite, nitrate and citrulline both in Kuppfer cells and the hepatocytes. It was subsequently shown that the supernatant from activated Kuppfer cells induced the formation of NO in the hepatocytes, an effect that was blocked by L-NMMA [257]. These data have led to the suggestion that Kupffer cells activated by septic stimuli or other inflammatory states respond by forming NO and by inducing NO formation in neighbouring hepatocytes. A major effect of NO is cytotoxicity due to suppression of hepatocyte protein synthesis. Induction of NO by endotoxin in hepatocytes has been demonstrated in vivo [258]. There is evidence that NO synthesis contributes to vasodilatation in portal hypertensive cirrhotic rats [259] and renal failure [260].

2.3 Evidence for role of nitric oxide in the biliary system

2.3.1 Nitric oxide and the gallbladder

As in the stomach, the gallbladder accommodates changing volumes with little change in pressure [261]. It is not clear whether this adaptive relaxation is neurally-mediated, or relies on passive compliance of the gallbladder wall. Mourelle

et al showed in anaesthetised guinea pigs and in isolated gallbladder muscle strips that resting gallbladder pressure and contractions induced by CCK were enhanced by L-NAME and abolished by sodium nitroprusside [262] and S-nitrosothiol [263]. A constitutive calcium dependent NOS was detected in gallbladder tissue homogenates [262].

It remains unclear whether NANC innervation is present in the gallbladder. Transmural field stimulation relaxed prairie dog ileal muscle but not gallbladder although muscle from both organs relaxed in response to S-nitroso-N-acetylpenicillamine (SNAP) with corresponding increases in cGMP concentrations [264]. NANC innervation could not be found in human gallbladder using EFS with adrenergic and cholinergic blockade [265]. In contrast, a similar study by McKirdy et al using EFS with a shorter pulse width, showed NANC inhibitory responses in 22 of 106 human gallbladder specimens [266]. Furthermore, this NANC relaxation was abolished by L-nitroarginine suggesting that NO could be the neurotransmitter involved. This is supported by evidence from an *in vivo* study in human that L-NMMA reduced the baseline gallbladder volume by about 15% and significantly augmented gallbladder emptying induced by CCK infusion and fatty meals [267]. Therefore it appears that NO relaxes gallbladder contraction, but it is not clear whether it is the mediator for NANC innervation in gallbladder.

2.3.2 Nitric oxide and the sphincter of Oddi

NANC neural relaxation is believed to be important in the mediation of the relaxatory response of the SO to CCK in humans [61] and the Australian opussum [268]. N^{G}-nitro-L-arginine methyl ester and oxyhaemoglobin both reduced the amplitude of relaxations induced by EFS in the presence of adrenergic and cholinergic blockade in isolated opussum SO precontracted with erythromycin; this effect could be partially reversed by L-arginine [269]. Sodium nitroprusside mimicked the relaxations induced by EFS. Furthermore histochemical staining of SO showed positive staining of NADPH-diaphorase in nerve cell bodies and nerve fibres in circular muscle [269]. Systemic infusion of L-NAME increased SO's tonic pressure and amplitude of

phasic contractions in guinea pigs and this effect was blocked by L-arginine [270]. CCK was known to contract the SO of guinea pigs and pre-treatment with L-NAME significantly enhanced this contraction; sodium nitroprusside antagonised the response to CCK [270]. Constitutive Ca^{2+} dependent NOS activity was present in fresh homogenates from guinea pigs and rabbit SO [270].

In humans, systemically administered gylceryltrinitrate has been demonstrated to lower SO basal pressure and contraction amplitude; and gallstones have been removed from intact papilla after glyceryltrinitrate induced relaxation [271,272]. Immunohistochemical labelling of human SO with anti-neuronal NOS showed abundant positive staining in nerve fibres and bundles [273]. Topical administration of S-nitroso-N-acetylcysteine (SNAC) reduced SO tonic pressure and decreased the frequency of phasic contractions [273].

2.4 The enzymology of Nitric oxide synthase

NO biosynthesis from arginine is carried out by a class of enzymes known collectively as the nitric oxide synthase (NOS). As might be expected on the basis of its diverse and ubiquitous physiology in a wide variety of cells and tissues, there is a wide variety of NOS isoforms [274]. All of the NOS isoforms characterised have common cofactors and prosthetic group requirements: NOS utilises O_2 and NADPH[275] and requires FAD, FMN [276] and tetrahydrobiopterin [277] for activity. They also have a strict requirement for L-arginine as D-arginine is a nonsubstrate. The mechanism by which the different isoforms convert arginine to NO and citrulline is likely to be the same as they all have common cofactors and prosthetic groups. However, apart from differences in tissue or cellular origin, the isoforms do differ with regard to their biophysical properties and in their mechanisms of regulation.

NOS can be divided into two distinct classes: an inducible class (iNOS) and a constitutive class (cNOS). Within each class, subclasses exist. There are at least two constitutive NOS with closely similar properties. One is the endothelial enzyme

(eNOS) and the other the neural enzyme (nNOS), with localisation to brain and peripheral NANC neurons.

Inducible NOS has been found in a wide variety of cells, for example, smooth muscle, hepatocytes, microglial cells, endothelium, neutrophils and macrophages. The iNOS in the macrophage differs from that in the endothelial cell, platelet and brain in that it is not detectable in macrophage cell lines or freshly elicited macrophages that have not been activated by lipopolysaccharide alone or in combination with γ-interferon [278] and it requires protein synthesis for its expression [277]. iNOS is different from cNOS in that it is functionally calcium independent. All reported cNOS isoforms are regulated by Ca^{2+} via the Ca^{2+}-binding protein calmodulin (CaM) [279]. A Ca^{2+} -CaM complex forms upon influx of Ca^{2+} into the cell, which then binds and activates cNOS. Thus, any event which results in the influx of Ca^{2+} into a cell containing cNOS can cause its activation and NO production. For example, acetylcholine, bradykinin or shear stress can cause influx of Ca^{2+} into endothelial cells and results in NO synthesis [274]. In contrast, the inducible enzyme from macrophages is not regulated by Ca^{2+} though it contains tightly bound CaM which can be considered an enzyme subunit [280]. Therefore in iNOS, CaM is always bound regardless of Ca^{2+} levels and its activity is not regulated by Ca^{2+}.

cNOS synthesises small amounts of NO on demands in response to agonists and is under stricter regulatory control than iNOS as the NO released is involved in maintenance of vascular tone and neurotransmission [274]. The agonists of cNOS are substances released form nerves, such as acetylcholine, bradykinin and excitatory amino acids, and those released from platelets e.g. thrombin, ADP and serotonin. Pharmacological activators are calcium ionophores [161,281]. In contrast to cNOS, iNOS releases large amounts of NO continuously; the amount released per unit of time from fully induced macrophages is a thousand times higher than that released from NOS in endothelial cells. The molecular targets in the victim cells are copper and

iron containing proteins, releasing free Cu^{2+} and Fe^{2+}, and generating O_2^- and highly toxic hydroxyl radicals resulting in lethal oxidative injury in the victim cells [171].

Table 2.1 Similarities and differences between the two NOS.

Constitutive	Inducible
NADPH dependent	NADPH dependent
Dioxygenase	Dioxygenase
Inhibited by L-arginine analogs	Inhibited by L-arginine analogs
Ca^{2+}/calmodulin dependent	Ca^{2+}/calmodulin independent
Picomoles NO released	Nanomoles NO released
Short lasting release	Long lasting release
Unaffected by glucocorticoids	Induction inhibited by glucocorticoids

Glucocorticoids inhibited the induction but not the activity *in vitro* of iNOS in endothelium and macrophages after stimulation with lipopolysaccharides, either alone or in combination with interferon [275]. The induction by lipopolysaccharides of the NOS in vascular tissues *in vitro* and the accompanying vascular relaxation, the hyporesponsiveness to vasoconstrictors, and the increase in cGMP were inhibited by incubation with dexamethasone [179]. This suggests that immunologically induced release of NO may indeed explain at least some of the pathophysiologically features of endotoxin shock. This action of glucocorticoids, which occurs at the low concentrations achieved in plasma during the therapeutic use of these compounds, correlates with their anti-inflammatory potency.

Considering the various mechanisms by which it is regulated and its requirement for so many cofactors and/or prosthetic groups, inhibition of NOS can be envisioned to occur in a variety of different ways. Inhibition of either iNOS or cNOS could occur by utilising agents which compete for L-arginine, NADPH, flavin or tetrahydropterin binding to the enzyme. Both cNOS and iNOS require NADPH-dependent FAD and FMN. Diphenyleneiodonium and its analogs are known inhibitors of NADPH-flavoproteins and were found to be potent inhibitors of iNOS from

macrophages and cNOS from endothelial cells [274]. Inhibition of tetrahydropterin biosynthesis can result in a loss of iNOS and cNOS activity in macrophages and endothelial cells [274]. As cNOS can be activated by Ca^{2+} via the reversible binding of CaM, CaM inhibitors calmidazolium was found to inhibit the endothelium cNOS dependent relaxation of rat thoracic aorta but had little effect on restoring the contractility of aorta with induced iNOS which was not dependent on CaM [282].

By far, analogs of arginine represent the largest and potentially most useful class of NOS inhibitors. Many of the previously mentioned inhibitors are inappropriate as *in vivo* inhibitors due to their non specific effects since many of these cofactors and prosthetic groups, for eg. NADPH, flavin, tetrahydropterin or CaM are utilised for other enzymatic reactions throughout the body.

A variety of arginine analogs have been found to be inhibitors of NOS activity. One of the most utilised and studied of these analogs is the methylated L-arginine analogue N^G-monomethyl-L-arginine (L-NMMA). The inhibition of NOS occurs by combination of competitive inhibition and irreversible inactivation of enzymes [274]. Other L-arginine analogues that have been described as NOS inhibitors in vascular tissues are N^G-nitro-L-arginine (L-NNA), its methyl ester L-NAME, and N-iminoethyl-L-ornithine (L-NIO) [179]. L-NIO is approximately five times more potent than the other analogues, suggesting that there may be differences in uptake, distribution or metabolism of these compounds.

As mentioned in previous sections, NOS has been identified by immunohistochemistry technique in a variety of tissues. Constitutive enzyme with a molecular weight of ~160 kDa has been purified from rat cerebellum [279]. The gene has been cloned and the deduced amino acid sequence showed that the molecule had recognition sites for NADPH, FAD, FMN and CaM [283]. Another cNOS has been isolated from native bovine endothelial cells and found to be of molecular weight ~135 kDa [284]. The inducible enzyme has also been purified to homogeneity from

murine macrophages [285] and further studies on cloning and sequencing of this enzyme showed 40% homology with the rat brain enzyme [286].

Antisera was raised in rabbits to rat brain enzyme and immunoreactivity was detected in brain, posterior pituitary, adrenal medulla, eye and intestine [187]. Two peptides were selected from the published sequence of the rat brain enzyme and synthesised for making the antibody [283]. These were peptide 49, corresponding to amino acids 251-270 and is situated close to the amino-terminal of neuronal NOS, and the carboxyl-terminal peptide 58, corresponding to amino acids 1409-1429. The antibody so raised showed strong reaction with the ~160 kDa protein in human brain extracts on Western blots [287]. In the human gut, staining was seen in the myenteric and submucous plexus, in both the neuronal cell bodies and fibres, and in nerve fibres within smooth muscle of the gut. Staining was seen in many areas of the human central nervous system, particularly cortex, hippocampus, hypothalamus, cerebellum, brain stem and spinal cord. Immunostaining with this antibody was also seen in the endothelium of blood vessels from human lungs, liver and skin [287]. The antibody reacted with enzyme of molecular weights between 125 and 140 kDa in these tissues. These could represent an endothelial form of the enzyme which is known to have molecular weights of 135 kDa [284]. Therefore, polyclonal antibodies raised to rat neuronal NOS recognise the neuronal form of the enzyme as well as other isoforms in the endothelium of human tissue. This finding indicates some degree of homology between the isoforms.

NADPH-diaphorase activity has been shown to be a reliable marker of neuronal NOS on the basis of its colocalisation with NOS-like immunoreactivity and NOS messenger RNA [189,288-290]. It has been shown that NOS activity is identical with NADPH-diaphorase in central and peripheral neural tissue [189,291]; also NADPH-diaphorase and NOS were shown to coexist in enteric neurone [235,292,293];

providing good evidence that NADPH-diaphorase staining reflects the distribution of NOS.

CHAPTER THREE

THE POST-CHOLECYSTECTOMY SYNDROME

3.1 Introduction

Gallstones affects 10 to 20% of the UK population and the incidence increases with advancing age. In 1993, cholecystectomy was performed at the rate of 1028.4 per million population in Scotland [294], making it one of the most common surgical procedures. However, not all cholecystectomy results in successful alleviation of symptoms. The term "post-cholecystectomy syndrome" has been used to describe a syndrome after cholecystectomy in which the pain is similar to that experienced by the patients before surgery.

Gallstones are usually "silent" [295-297] or infrequently become symptomatic [298]. Several studies have demonstrated that most persons with minimally symptomatic or "silent" gallstones remained free of symptoms and serious complications for prolonged periods and therefore do not require specific therapy [299-301]. The cumulative probability of developing biliary colic after 10 years was 15% in the study by Gracie and Ransahoff [299] and 25.8% in the GREPCO Study [302]. Ransahoff and colleagues suggested that prophylactic cholecystectomy for asymptomatic gallstones was unnecessary [303]. In contrast, patients who had experienced biliary type pain usually continued to have recurrent symptoms [301] or went on to develop complications [304]. Wenckert and Robertson [304]found that a third of patients developed severe disabling biliary pain and a fifth acute cholecystitis or other serious complications over an 11 years period. Patients who develop biliary colic or acute cholecystitis should therefore be treated by cholecystectomy.

3.1.1 Historical data from open cholecystectomy.

Studies that have assessed symptomatic outcome after open cholecystectomy have given varying results for the incidence of persistent pain after cholecystectomy. Studies from the 1960's suggested that the incidence of postcholecystectomy symptoms was between 18 to 43 percent [305-307]. In the 1980's, Ros and Gambon evaluated 93 patients before and two years after cholecystectomy [308]. Only 53 patients were completely symptom free at two years. The authors observed that patients with a shorter duration of symptoms before cholecystectomy achieved a

higher cure rate than those with a longer preoperative history [308]. Through retrospective case notes review and questionnaires sent to patients' general practitioners, Konsten et al [309] reported an incidence of symptoms of 18% at a median interval of 10 years after cholecystectomy. The authors also noted that patients with typical symptoms of gallstone disease before surgery had significantly fewer complaints during follow-up than those with atypical symptoms. Typical biliary pain was defined by the Rome working group report [310] as colicky pain in the right upper quadrant of the abdomen. Gilliland and Traverso followed up over 600 patients over a mean follow up of 45 months and found that 88% of patients had complete symptomatic relief after cholecystectomy [311]. It was again noted that patients without the pain of biliary colic were less likely (75%) to obtain symptom relief from cholecystectomy. Dyspepsia was the most common residual symptom after cholecystectomy in this subset, occurring in 32 percent.

The varying incidence of postcholecystectomy syndrome can be ascribed to the varying methods of evaluation, symptoms being assessed and case mix of patients' indications for surgery. Words such as "dyspepsia" and "flatulence" mean different things to many patients. Flatulent dyspepsia has been defined as "epigastric discomfort after meals, a feeling of fullness so that tight clothing is loosened, eructation with temporary relief, and regurgitation of sour fluid to the mouth with heartburn". Clearly, this is a rather loose definition as many of the features are also found in patients with gastroesophageal reflux disease or peptic ulcer disease.

3.1.2 Incidence from laparoscopic cholecystectomy

There have been few studies on the symptomatic outcome following laparoscopic cholecystectomy. In a 3-month follow-up of 52 patients, Peters et al reported that 77 percent of patients considered their symptoms to be cured [312]. In a comparative study of open and laparoscopic cholecystectomy, Vander Velpen et al reported that 95 percent of patients considered that they had obtained overall symptomatic improvement [313]. Qureshi et al analysed perceptions of postoperative symptoms and global satisfaction [314]. They noted that 25 percent of patients complained of more

than two symptoms postoperatively but 84 percent considered the procedure to be a complete success. Wilson and Macintyre evaluated symptomatic outcome of 115 patients a year after laparoscopic cholecystectomy and compared the outcome with that in 200 patients who had undergone the open procedure. Over 90 percent of patients in both groups considered the procedures to be successful [315].

3.1.3 Causes of post-cholecystectomy pain

Upper abdominal pain is the major symptom of gallstone disease [295,298,316-318] but the quality, duration, periodicity and frequency of pain most characteristics for cholelithiasis remain uncertain [317-323], even in patients presenting with acute upper abdominal pain [324]. It is difficult to be certain in any one patient whether the abdominal pain is due to gallstones or other upper abdominal pathologies as all may produce similar symptoms. Incorrect preoperative symptom assessment may be an important reason for poor outcome in cholecystectomised patients.

The majority of patients with the post-cholecystectomy syndrome have symptoms originating from the oesophagus, stomach or duodenum. Gastroscopy and barium meals may identify these diseases. Other patients have functional bowel disorders such as irritable bowel syndrome or non-ulcer dyspepsia. Meticulous assessment is therefore required before cholecystectomy.

Only relatively small number of patients with postcholecystectomy pain have biliary tract diseases such as common bile duct stones, ampullary tumour or sphincter of Oddi dysfunction [325].

3.2 SO dysfunction

SO dysfunction has been estimated to account for 1 to 10% of patients with post-cholecystectomy pain [326]. The possible aetiologies are discussed in 10.4.

3.2.1 Clinical characteristics

SO dysfunction continues to be a poorly defined syndrome despite significant progress in endoscopy and manometry. Many such patients have undergone cholecystectomy without discernible abnormalities in the gallbladder. There is a preponderance of middle aged women. Abdominal pain remains the most common presenting symptom occurring a variable time after cholecystectomy. The pain is similar to the one before surgery and is usually located at epigastrium or right upper quadrant and may radiate to the back or shoulder blade. It is usually precipitated by food and may be associated with nausea or vomiting. Jaundice and cholangitis are rarely observed [325]. Physical examination is usually unremarkable. There may be a transient rise in liver function tests such as alkaline phosphatase, ALT or GGT during attacks of pain. Some patients may present with acute recurrent pancreatitis.

3.2.2 Classification

SO dysfunction is classified as fixed structural stenosis or transient dyskinesia. Stenosis is thought to be caused by the passage of gravel and surgical instrumentation, with resulting oedema, fibrosis and a fixed narrowing of the SO that may fail to respond to a variety of neural, physiologic or pharmacologic stimuli. SO stenosis is defined manometrically as fixed, elevated basal sphincter pressure (>40mmHg) that is unresponsive to CCK or amylnitrate. SO dyskinesia consists of three motor abnormalities: (1) transient elevation of basal sphincter pressure (>40mmHg) which relaxes to CCK or amylnitrate, (2) increased number of phasic contractions (>7 per minute) and (3) increased percentage of phasic contractions that propagate retrogradely (see table 5.4). Geenen and Hogan classified SO dysfunction as type I to III (table 3.1 and 3.2) [327,328]. This classification separates patients into subgroups based on a number of clinical, laboratory and radiographic findings. This classification has been criticised for being empirical and lacks discrimination for patients subsets. It has only been validated for patients with previous cholecystectomy. It is cumbersome and impractical to determine delayed drainage of contrast from the biliary tree, which is one of the scoring criteria. The proponents argue that it forms a useful common standard by which differrent studies can be compared [329].

Table 3.1 Sphincter of Oddi dysfunction: Biliary type.

Group 1	Recurrent biliary pain.
	Twofold elevation in aspartate aminotransferase or alkaline phosphatase
	Prolonged common bile duct drainage time (>45 minutes)
	Dilated bile duct(>12mm)
Group 2	Biliary-type pain plus 1 or 2 of Group 1 criteria
Group 3	Biliary-type pain only

Table 3.2 Sphincter of Oddi dysfunction: Pancreatic type

Group 1	Recurrent pancreatitis or recurrent pain with amylase or lipase elevation
	Twofold elevation in aspartate aminotransferase or alkaline phosphatase.
	Dilatation of pancreatic duct (>5mm head,>4mm body)
	Prolonged pancreatic duct drainage time (>9 minutes)
Group 2	One or two of group 1 criteria
Group 3	Pancreatic-type pain with normal enzymes and pancreatogram

3.2.3 Investigations

Non-invasive diagnostic investigations of patients with suspected SO dysfunction can be performed by the morphine-prostigmine test, quantitative hepatobiliary scintigraphy (QHBS) and the fatty meal scintigraphy (FMS).

(1) Morphine-prostigmine test

Intramuscular injection of morphine and neostigmine is followed up to 5 hour by determination of liver function tests. The test is considered positive if pain is reproduced and liver enzymes increase to more than two times the upper normal limit. Several studies have shown that sensitivity and specificity are poor [330].

(2) Fatty-meal sonography

Common bile duct (CBD) diameter is generally regarded to be slightly dilated after cholecystectomy as it assumes a reservoir function and 7 mm is regarded as the upper limit of normal as measured ultrasonographically [327]. CBD measurement is made before and 45 minutes after ingestion of a fatty meal (Lipomul 1.5 mL/kg, Mead Johnson Laboratories). CCK released after the meal stimulates increased hepatic flow, contraction of the gallbladder (if present) and relaxation of the SO. Darweesh et al defined a positive response as increase in the diameter of the CBD of more than 2mm [328]. Using these criteria, a sensitivity of 74% and specificity of 100% for partial CBD obstruction was obtained. Hepatocellular diseases do not affect the FMS.

(3) Quantitative hepatobiliary scintigraphy

The test involves a large field-of-view gamma camera and administration of technetium^{99m} labelled radiopharmaceuticals that are actively taken up by hepatocytes and excreted into the biliary tree. The most common radiopharmaceuticals used are Tc^{99m}-HIDA (dimethyl-iminodiacetic acid), Tc^{99m}-EHIDA (2,6 diethylphenylcarbamoylmethyl diacetic acid), Tc^{99m}-disofenin and Tc^{99m}-mebrofenin. Darweesh et al reported reduced hepatic clearance (<63%) at 45 minutes as the most sensitive indicator for partial CBD obstruction with 67%

sensitivity and 85% specificity [331]. The test cannot be used in the presence of hepatocellular diseases.

FMS and QHBS were initially thought to show great promise in screening for SO dysfunction but they do not differentiate SO dysfunction from other causes of distal common bile duct obstruction. When the FMS or QHBS are positive, ERCP with or without manometry should be considered to differentiate SO dysfunction from other causes of distal CBD obstruction such as benign stricture, choledocholithiasis or tumour. FMS and QHBS have recently been reported to be poorly correlated with manometry and the diagnostic accuracy decreases from Type I to Type III [332].

(4) Endoscopic Retrograde Cholangiopancreatography

ERCP demonstrates morphologic abnormalities. It is an essential investigation before manometry as endoscopic evaluation of the papilla and peripapillary area can reveal abnormalities such as papillitis, choledochocele and tumour. ERCP demonstrates choledocholithiasis and tumours of the biliary tract. In SO dysfunction, the biliary tree can appear normal. However, the following radiographic abnormalities may occur: (1) dilatation of CBD to more than 12mm. This is the most common radiographic abnormality noted among patients with SO dysfunction, particularly in type I biliary dyskinesia. (2) delayed drainage of contrast from CBD (>45 minutes), (3) dilatation of pancreatic duct (>6-7mm), (4) delayed drainage of the pancreatic duct (>9 minutes) and (5) narrowing of the distal CBD with difficulty of cannulation.

SO manometry remains the gold standard for the diagnosis of SO dysfunction. However, the technique requires extensive experience of ERCP and manometry. This is discussed further in section 5.3.

CHAPTER FOUR

STATEMENT OF AIMS OF THESIS

4.1 Introduction and aims

Review of the literature reveals many unanswered questions relating to gallbladder and SO function. The particular aims of the work described in this thesis are as follows.

1. To define the mechanisms of early gallbladder emptying. It has been known for many decades that CCK is released from small intestinal mucosa in response to intraluminal fat and protein [83,86], and that CCK stimulates gallbladder contraction and SO relaxation [4,5]. This classical physiological pathway does not however explain the observation that the gallbladder starts to empty immediately after ingestion of food, many minutes before gastric emptying occurs and nutrients enter the duodenum. The mechanism for the early phase of gallbladder emptying is unclear although it is inhibited by atropine. Fisher et al [26,333] and Hansen et al [334] both demonstrated that sham feeding caused gallbladder emptying in man and that this phase of gallbladder emptying was inhibited by atropine and vagotomy. It therefore appears that a vagal cholinergic reflex could be responsible for this early phase. Alternatively, it is possible that gastric secretions produced by cephalic and oral stimulation, and gastric distension may enter the duodenum and initiate gallbladder emptying via CCK released from enterocytes. The first part of the thesis therefore investigated the mechanism of this early phase of gallbladder emptying.

2. To determine whether NO influences gallbladder emptying and SO motility. The enzyme NOS is responsible for the formation of NO from the amino acid L-arginine and the activity of this enzyme is inhibited by an analogue of L-arginine, L-N^G-monomethyl-arginine (L-NMMA) [158]. There is much evidence to show that the L-arginine : NO pathway is an important component of NANC neurotransmission in the lower oesophageal sphincter [205], colon and internal anal sphincter [246]. The possible role of NO upon gallbladder motility is unexplored. In a study of electrical field stimulation, sparse inhibitory NANC activity was identified in isolated human gallbladder muscle strips[266]. However, NO has not been systemically studied. The effect of NO upon gallbladder emptying and SO motility was examined.

3. To investigate SO motility before and after LC. Upper abdominal pain after cholecystectomy is a well recognised phenomenon. It is sometimes attributed to SO dysfunction after exclusion of biliary stones and strictures. The syndrome is well described by Geenen et al [325]. The majority of patients with objective signs of disturbed bile flow (elevated liver enzymes, dilated common bile duct and delayed drainage of contrast during ERCP) also have abnormal manometry. However, in these studies SO manometry was performed only in patients after cholecystectomy and it is unknown whether biliary dyskinesia is a consequence of denervation incurred during cholecystectomy. Denervation could disrupt CCK responsive descending inhibitory nerves and thereby leave CCK mediated smooth muscle contraction unopposed. We therefore investigated prospectively SO motility before and after laparoscopic cholecystectomy (LC).

4. To determine the incidence of post-cholecystectomy syndrome. A small number of patients continue to have a variety of gastrointestinal symptoms following LC. The term "post-cholecystectomy syndrome" has been used to describe this condition. The reported incidence of post-cholecystectomy syndrome varies widely. Studies on the symptomatic outcome after LC have reported successful relief of symptoms in 70% [312,314] to 95% of patients [313,315] but the characteristics of patients who continue to experience pain after surgery are poorly defined.

LC has almost completely replaced open cholecystectomy but the incidence of persistent pain after LC is poorly documented. We therefore prospectively assessed the incidence of the LC syndrome 6 months after surgery using standard questionnaire.

4.2 Plan of investigation

Firstly, the sensitivity and reproducibility of ultrasonic measurement of gallbladder volume were determined (see 4.2). The coefficient of variation of ultrasonic measurement of gallbladder volume was calculated. As the study involved repeated measurements of gallbladder emptying in the same individuals, reproducibility of gallbladder emptying after standard meals was determined.

As gallbladder smooth muscle contraction depends on the menstrual cycle, only male volunteers were chosen for gallbladder emptying studies. The mechanism of the early phase of gallbladder emptying was examined by subjecting volunteers to ingestion of fatty food, modified sham feeding and gaseous gastric distension. Gallbladder volumes, plasma immunoreactive CCK and gastrin concentrations were measured.

The role of nitric oxide in gallbladder smooth muscle contraction was examined *in vitro* using isolated bovine and human gallbladder muscle strips. The strips were set up in water baths; contraction and changes in muscle tension were measured using isometric transducers. The muscle strips were contracted with CCK and the effect of NO examined by adding NO donors (glyceryltrinitrate and sodium nitroprusside). Human gallbladders were stained immunohistologically for NOS. This was followed by an *in vivo* experiment involving a group of healthy male volunteers. Gallbladder emptying after fatty meal was observed during intravenous infusion with normal saline, NO donors and hydralazine as a hypotensive control agent.

SO motility was studied manometrically. Patients undergoing SO manometry for investigation of abdominal pain were enrolled into the study for the effects of nitric oxide on SO motility. After the recording of motility indices, glyceryltrinitrate was applied topically on the ampulla and further recording was obtained for further two minutes. Another group of patients awaiting LC for cholelithiasis volunteered for the experiment examining the changes of SO motility caused by cholecystectomy. SO manometry was performed a few days before and six months after LC.

CHAPTER FIVE

METHODOLOGY

5.1 Measurements of gallbladder emptying

In experimental models, gallbladder motility can be measured by directly cannulating the intact gallbladder and measuring the intraluminal pressure. Isolated gallbladder tissue can be obtained from either patients or experimental animals and contraction assessed in organ baths with transducers. Alternatively gallbladder emptying can be assessed *in vivo* either by serially measuring gallbladder volumes using cholecystography or sonography, or by measuring the turnover rate of bile in the gallbladder by scintigraphy or aspiration of bile through an indwelling catheter placed in the duodenum [335,336].

5.1.1 Scintigraphy

Scintigraphy involves radioisotope scanning over the gallbladder after the administration of a radiolabelled cholecystographic agent such as ^{99m}Tc-HIDA [337,338]. Provided hepatic functions is normal, ^{99m}Tc-HIDA is rapidly excreted in bile when administered intravenously. Serial images are obtained as the isotope accumulates in the gallbladder. After correction of natural decline in radioactivity over time, the decay of radioactivity in minutes over gallbladder represents changes in gallbladder volumes. It monitors the kinetics of gallbladder filling and emptying and measures changes in gallbladder volumes through changes in radioactivity. It does not however measure changes in absolute gallbladder volumes. Its usefulness is limited by the need for fairly complex equipment and by radiation exposure. Therefore repeated measurements may be hazardous during experimental conditions.

5.1.2 Aspiration method

The aspiration method involves aspiration of bile from the duodenum and the use of nonabsorbable markers which facilitate calculation of bile acid and bilirubin output [339]. The rate and duration of gallbladder emptying can be calculated but gallbladder volumes or extent of emptying cannot be ascertained. It is an invasive method and uncomfortable method which cannot be applied repeatedly.

5.1.3 Ultrasound

With the use of newer high resolution sector real time ultrasound systems, sonography has replaced cholecystography in the imaging of biliary systems in clinical practice for many years. It identifies stones, sludge and wall thickening. It can be performed rapidly and repeatedly without radiation or contrast administration. Absolute volumes and extent of emptying can be measured. It is simple, safe and well tolerated technique yielding rapidly acquired data.

(1) Methods

The measurement of gallbladder volume by a geometric method, called the sum of cylinders method, was originally described in 1948 by de Silva using cholecystography. The method assumes that the gallbladder is a pear shaped organ that can be cut into a series of small cylinders of equal heights (h) that can be stacked one above the other. A grid is placed on the optimal longitudinal gallbladder image so that the lines spaced at 1 cm apart are oriented at 90^0 to the longitudinal gallbladder axis. When the gallbladder is curved, as it often is, the longitudinal axis is displaced from the central axis (figure 4.1). This causes the diameter measured from the longitudinal projection to be smaller than the true diameter of the gallbladder (figure 5.1). Everson et al modified de Silva's method slightly by applying the correction factor (E) to account for the change in curvature of the gallbladder [335].

The formula for gallbladder volume is :

$$V = 0.785hE^2 \left(\sum_{i=1}^{n} di^2 \right)$$

where h and d are the respective height and diameter of the ith cylinder and n represents the total number of cylinders.

(2) Limitations.

It is assumed that the displacement of the longitudinal projection from the central axis is uniform throughout the length of the gallbladder. This is not always true, especially

in long, narrow tapering segments of the gallbladder, where volume is small. The volume in these areas would be over estimated slightly, but the effect on total volume is minimal. The gallbladder also has segmentations and septa which influence the calculation of volume by the geometric method.

The sum of cylinders method has been used effectively in investigative studies of gallbladder volume in health and disease [34,340]. It gives a good approximation of gallbladder volume but requires computational software in the calculation of volume and this option is not generally available. Dodds et al subsequently compared gallbladder volumes calculated by the simple ellipsoid method with the sum of cylinders method and showed that the two methods give comparable values [341]. The formula for the ellipsoid method is :

$$V=\pi/6\ (L \times W \times H)$$

where L is the length, W is the width, and H is the height.

The work in this thesis employed this ellipsoid method.

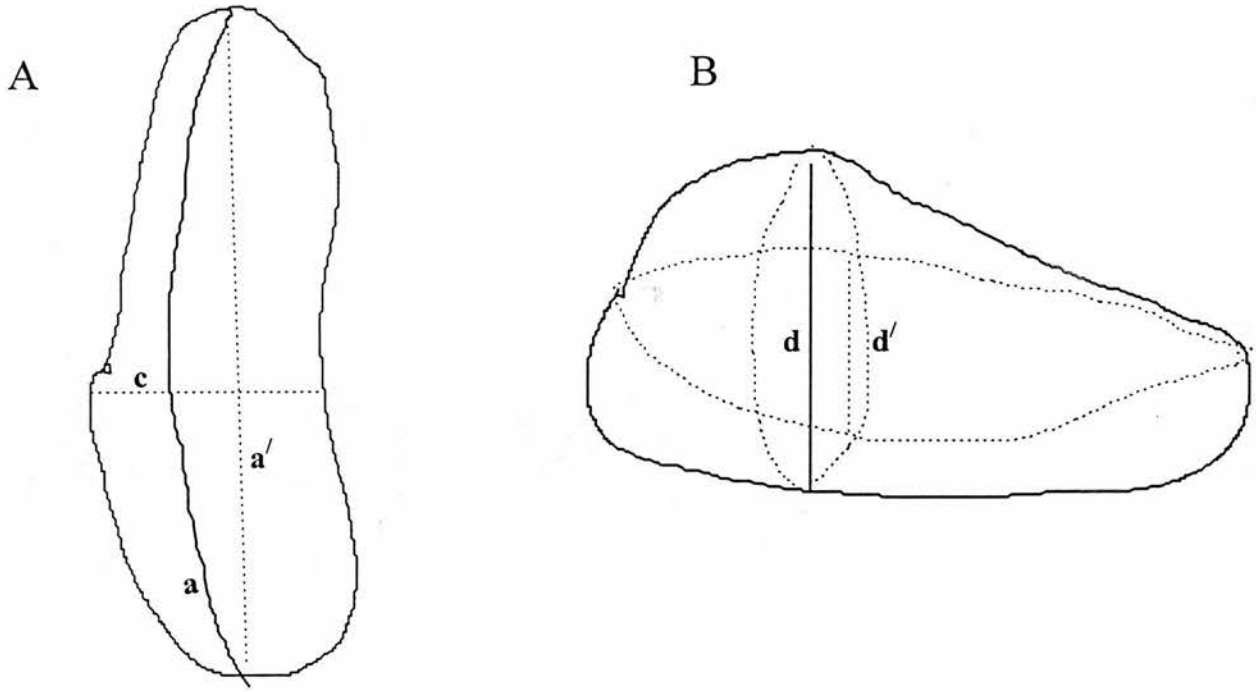


Figure 5.1 A. Diagram showing relationship of "a", central axis of the gallbladder, "$a^{/}$" longitudinal axis from the sonogram, and "c" short axis from the sonogram. B. Sketch of ultrasound longitudinal projection (----) superimposed upon actual size(━) of gallbladder. The short axis projection is also shown with diameter "d" and "d^{1}". d^{1} is the diameter which is measured from the sonographic longitudinal long axis.

5.2 Reproducibility of gallbladder emptying rates and intra-observer coefficient of variation of ultrasound scans.

Some studies described in this thesis involved repeated measurement of gallbladder emptying rates in the same individuals. Therefore day to day variations in fasting gallbladder volumes and emptying rates, and the intra-observer accuracy in determining gall bladder volumes by ultrasound were determined.

5.2.1 Methods

10 healthy volunteers (5 male, 5 female; median age 36.5 years, range 23-52) were studied on two separate mornings after overnight fast. Images of gallbladder volumes were obtained by a portable real time ultrasound system (Sonoline SX, Siemens, Germany). The transducer was placed in a sagittal plane in the right upper quadrant of the supine volunteer and positioned until the greatest length (longitudinal axis) of the gallbladder was obtained. The image was then frozen on the oscillocope screen, and the dimension (length) was measured on the screen with electronic calipers. The transducer was then rotated 90^0 to obtain the image of the transverse axis of the gallbladder until the greatest width and height dimensions were obtained. All measurements were made from the inner gallbladder wall. In some volunteers, the gallbladder was located high under the right subcostal border and a deep, held inspiration was necessary for visualisation. The part of the oscilloscope screen showing the measurements was covered with a screen away from the ultrasonographer and the reading was noted by an assistant.

None of the subjects had gallstones. Pre-menopausal female volunteers were examined during the luteal phase of their menstrual cycle. Smokers were requested to refrain from smoking for at least six hours prior to scanning. None of the volunteers was taking motility drugs. Each subject was requested to undergo the study on two separate mornings.

The intraobserver i.e. intra-study, coefficient of variation was estimated by making five measurements of fasting gall bladder volumes over a period of 10 minutes. It was

assumed that fasting gallbladders remained static over the 10 minute period. Intra-observer coefficient of variations of gallbladder volume were then calculated (SD (standard deviation) over the means of the fasting volumes multiplied by 100) for each individual subject. Inter-study (between day 1 and day 2) and inter-subject (for day 1 and 2) were determined from the fasting gallbladder volumes.

To determine day to day variation in gallbladder emptying, volunteers were requested to ingest a standard fatty meal comprising a two egg omelette. Gallbladder volumes were measured at 5 minute intervals for the first thirty minutes, at 10 minute intervals for the second thirty minutes and at 15 minute intervals for the final thirty minutes. Duplicate measurements were made at each time interval and the average volumes calculated. Gallbladder volumes were expressed in millilitre and as percentage of fasting volume at interval after ingestion of fatty meal. Fasting volumes, residual volumes and percentage of fasting gallbladder volumes at each time interval were noted.

To test for reproducibility of the rate of gallbladder emptying on two different days under similar experimental condition, interstudy coefficient of variations at each time intervals of the mean percentage of fasting gallbladder volumes were determined.

Written informed consent was obtained from each volunteer and the studies were approved by the Lothian Ethical Subcommittee for Medicine and Oncology.

5.2.2. Results

Intra-study coefficient of variation

The means $\pm$ SD of fasting volumes and intra-observer/intra-study coefficient of variations for each subject are shown in table 5.1. Intra-observer coefficient of variation in ultrasound measurement of gallbladder volume was below 12. The mean intra-observer/intra-study coefficient of variation was found to be 7.7 (Range 3.6-11.3).

Inter-subject coefficient of variation

The variation in fasting gallbladder volumes between subjects was wide, ranging from 4 to 30ml . Mean±SD of fasting gallbladder volumes for the group on day 1 and 2 were 19.1±6.5 and 21.7±8.6 ml respectively (table 5.1). The inter-subject coefficient of variations for the two days were therefore (6.5/19.1 x 100= 34) and (8.6/21.7 x 100= 39.6). This wide variation in gallbladder volumes is well known and has been found by other investigators [41,342].

Inter-study coefficient of variation

As shown in table 5.2, the mean of the difference (Day 1- Day 2) in fasting gallbladder volumes on two separate days were 3.2±3.4. The mean of fasting gallbladder volumes from the two days was (19.1+21.7)/2= 20.4 ml. The inter-study coefficient of variation could be calculated from the SD of the difference divided by the mean of fasting gallbladder volumes. This inter-study coefficient of variation was found to be (3.4/20.4x100)= 16.7.

The mean fasting and residual gallbladder volumes for the whole group, and fasting gallbladder volumes for the male and female volunteers are shown in table 5.3. The mean fasting volumes were 19.1±6.5 ml and 21.7±8.6 ml on the first and second day respectively. Male volunteers had significantly larger fasting gallbladder volumes than female. The mean fasting volumes were 15.7±5.7 and 27.6±3.9 ml for the female and male volunteers respectively (unpaired t test, $p<0.05$).

Reproducibility of the rate of gallbladder emptying

Profiles of gallbladder emptying after standard fatty meals for the two days are shown in figure 5.3. The difference between day 1 and day 2 in the percentage of fasting gallbladder volumes at each time interval for each individual, and the means and standard deviations of the difference, is shown in table 5.4. The means of percentage of fasting gallbladder volumes at each time interval for the whole group is shown in table

5.5. The coefficient of variations for gallbladder emptying at similar time interval on two different days was then calculated by dividing the standard deviations of the difference in the percentage of fasting gallbladder volumes by the means for the whole group. As shown in table 5.5, the coefficient of variations ranges from 4.9-15.4. Apart from three time intervals at 40th, 50th and 60th minute, the values are otherwise below 10. This result confirms the visual impression of figure 5.3 showing that the rate of gallbladder emptying is reproducible under similar experimental condition.

Table 5.1 Mean fasting gallbladder volumes (five repeated measurements per minute and intra-observer coefficient of variation of ultrasound scan measurements).

Subject	**Sex**	**Mean fasting volume±SD ml (day 1)**	**Mean fasting volume±SD ml (day 2)**	**Coefficient of variation (day 1)**	**Coefficient of variation (day 2)**
1	female	7.1±0.4	4.6±0.5	5.6	10.8
2	female	13.1±1	14.5±1.1	7.6	7.6
3	female	13.4±1.5	13.2±0.7	11.2	5.3
4	female	21.6±2.2	24.8±1.5	10.0	6.1
5	female	19.7±2	21.3±1.4	10.2	6.5
6	male	18.4±1.2	30.5±2.4	6.5	7.9
7	male	19±1.9	22.1±2	10	9.5
8	male	24.8±0.9	25.1±1.2	3.6	4.8
9	male	26.2±1.7	29.8±2.8	6.5	11.3
10	male	27.8±1.9	30.7±2.2	6.8	7.2
Mean fasting volumes for the group±SD ml		19.1±6.5	21.7±8.6		

Table 5.2. Means of fasting gallbladder volumes and the difference between day 1 and day 2.

Subject	Sex	Mean fasting volume±SD ml (day 1)	Mean fasting volume±SD ml (day 2)	(Day 1-Day 2) of fasting gallbladder volumes
1	female	7.1±0.4	4.6±0.5	2.5
2	female	13.1±1	14.5±1.1	1.4
3	female	13.4±1.5	13.2±0.7	0.2
4	female	21.6±2.2	24.8±1.5	3.2
5	female	19.7±2	21.3±1.4	2.2
6	male	18.4±1.2	30.5±2.4	12.1
7	male	19±1.9	22.1±2	3.1
8	male	24.8±0.9	25.1±1.2	0.3
9	male	26.2±1.7	29.8±2.8	3.6
10	male	27.8±1.9	30.7±2.2	2.9
Mean of the (Day 1 - Day 2) in fasting gallbladder volumes ±SD				3.2±3.4

Table 5.3 Mean fasting and residual gallbladder volumes for the whole group.

	Mean±SD
Day 1 Fasting GB Volume (ml)	19.1±6.5
Day 2 Fasting GB Volume (ml)	21.7±8.6
Day 1 Residual GB Volume (ml)	4.5±3.7
Day 2 Residual GB Volume (ml)	3.1±2.2
Mean fasting volumes for female subjects (ml)	15.7±5.7
Mean fasting volumes for male subjects (ml)	27.6±3.9

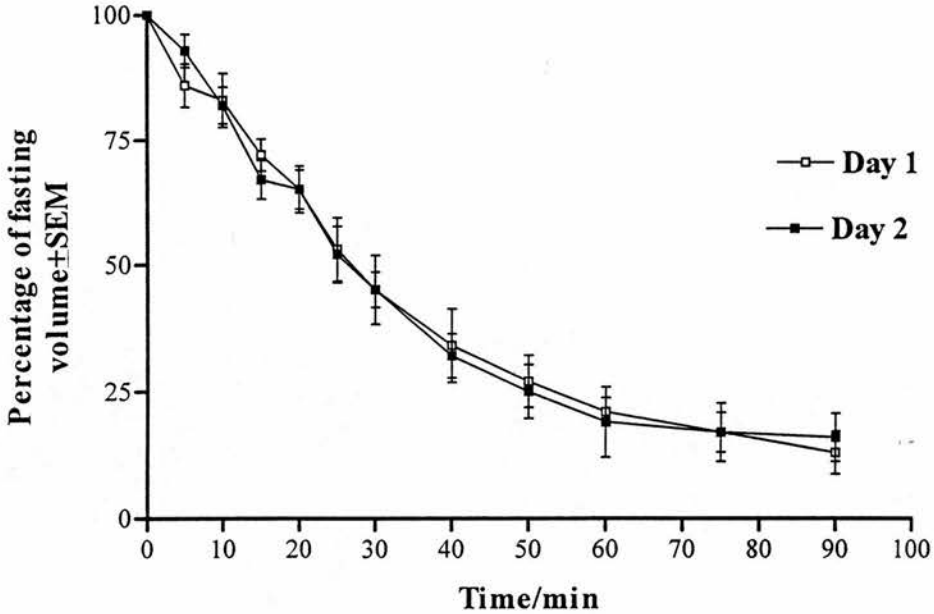


Figure 5.2 Gallbladder emptying rates on two different days after ingestion of fatty meal in ten subjects.

Table 5.4. (Day 1 - Day 2) in the percentage of fasting gallbladder volumes at different time points for individual subject and the means for the group.

Time/ min	(Day 1-Day 2) of the percentage of fasting gallbladder volumes for individual subject										Means±SD
	1	2	3	4	5	6	7	8	9	10	
0	0	0	0	0	0	0	0	0	0	0	0
5	2	2.3	12.4	4.5	20	2	10	11	14.4	7.2	8.7±6.4
10	11	19.5	18.5	9.6	9	6	18.4	8	13.2	11.3	12.6±5.1
15	6	15.7	13.1	11.7	5	1	10.8	3	3.8	7.2	7.8±5.1
20	5	6	5.8	17.8	13.1	4	11.9	11	12.1	8.9	9.6±4.6
25	19	5.8	11.9	10.9	3.9	3	8.8	8	8.1	8.3	8.8±4.8
30	1	3.6	3.6	12.1	7.7	4	4.3	5.0	10.1	9.8	5.6±3.5
40	8	12.5	3.2	15	17.4	1	3.0	2.0	1.5	6.9	7.1±5.4
50	8	2.7	3.4	10.7	3.3	1	0.1	2	5.4	1.7	3.6±3.7
60	52	1.3	4.1	2.7	1.9	4.3	5.4	1	8.8	4.8	3.5±2.4
75	62	0.2	0.1	1.6	2.0	0	1.8	1.1	1.4	2.7	1.1±0..8
90	73	0	0.8	0.9	3.9	1	1.2	0.3	0.3	3.4	1.3±1.3

Table 5.5 Means of fasting gallbladder volumes on day 1 and 2, standard deviations of the difference in percentage of fasting gallbladder volumes and the coefficient of variation at each time intervals.

Time/min	Means of percentage of fasting gallbladder volumes			SD *	Coefficient of variations
	Day 1	Day2	Means of day 1 and 2		
0	100	100	100	0	0
5	91.2	84.4	87.8	6.4	7.3
10	79.9	81.7	80.8	5.1	6.3
15	67.1	73.7	70.4	5.1	7.2
20	63.7	63.9	63.8	4.6	7.2
25	51.9	51.9	51.9	4.8	9.2
30	44.8	45.8	45.3	3.5	7.7
40	32.9	33.3	33.1	5.4	13.3
50	24.1	23.9	24.0	3.7	15.4
60	18.7	18.6	18.7	2.4	12.8
75	16.3	16.3	16.3	0.8	4.9
90	15.6	16.1	15.8	1.3	8.2

* SD is the standard deviation of the (Day 1 - Day 2) of the percentage of fasting gallbladder volumes at each time point for the group (see last column on table 5.4).

5.3 SO manometry

SO manometry is the gold standard test for SO dysfunction. However, it is invasive and the technique requires extensive endoscopic experience. The manometry equipment is not generally available in most hospitals. Recording and interpretation of manometric tracing requires computational software and a highly experienced observer.

5.3.1 Water perfusion technique

(1) Methods

After overnight fast, patients were sedated with 5-10 mg intravenous midazolam. This agent has been reported to have no effect on SO motility [343]. Opiate related drugs and glucagon were avoided as they interfere with SO motility. Patients were given ciprofloxacin 500 mg two hours prior to SO manometry. The Olympus JF1T10 side viewing endoscope was introduced into the stomach and advanced into the duodenum with the patient in the left lateral position. Patients were then turned onto the prone position prior to cannulation of the ampulla.

A triple-lumen polyethylene manometric catheter with an external diameter of 1.7 mm, luminal diameter of 0.5 mm and a length of 200 cm was used. It had three lateral openings, 0.5 mm in diameter, located at 2 mm intervals. The most distal end of the catheter was marked by eight black rings 2 mm apart to permit endoscopic observations of the depth of catheter insertion into the ampulla.

For pressure recording, each catheter lumen was perfused continuously with degassed sterile water by a low-compliance pneumohydraulic capillary infusion pump at a flow rate of 0.25 ml/min (Arndorfer Medical Specialities) This was connected via a transducer to a computerised polygraph (Albyn Medical Ver.6.0, England). Transducers were calibrated before each study to produce a pressure rise in excess of 250 mmHg/sec when occluded.

After recording duodenal pressure, taken as the zero reference, the catheter was deeply cannulated into the CBD. Pressure was recorded and the catheter was then withdrawn across the SO in 2 mm increments using the black marks on the catheter as a guide. The sphincter was identified as the zone of high resting pressure between the duct, either pancreatic or choledochal, and the duodenal pressure with phasic waves superimposed. Recording was obtained for at least 60 seconds at each station. Ideally, the three tips should be recording phasic wave contractions simultaneously. As soon as this occurred, the catheter was kept stationary at this position.

After at least two such pull-throughs, the catheter was repositioned so that all three channels recorded phasic SO contractions. CCK (Cholecystokinin, Ferring Pharmaceuticals, Malmo, Sweden) was injected intravenously at 1 IDU/kg bodyweight over 60 seconds. After 30 seconds period to allow for equilibration of drug concentration, recordings were continued for 2-3 minutes. The total duration of recording lasted approximately 10 min. Selective bile duct cannulation was facilitated by the careful positioning of, and direction of, the catheter relative to the papilla. Other investigators perform radiological imaging to confirm catheter position in either the CBD or pancreatic duct. This needs a radiopaque catheter to determine whether the catheter advances towards the liver hilum in the CBD or leftward in the pancreatic duct [344]. Otherwise injection of contrast is needed. Distension of the biliary tree is known to alter SO motility [69,345]. Guelrud et al verified catheter position by aspirating through the distal port before recording; aspiration of bile is believed to indicate the position in the CBD whereas aspiration of clear colourless fluid indicates the pancreatic duct [346].

In our studies verification was not performed because a facility for radiological screening was unavailable. Furthermore SO motility were found to be similar for CBD and pancreatic intubation [346,347].

Ductal pressure was known to show biphasic variations with respiration, rising on inspiration and falling on expiration [344]. Pressure varies in relation to posture.

Duodenal, CBD and pancreatic duct pressures were found to be significantly higher in the prone than in the left lateral position, probably because of the influence of intra-abdominal pressure [344]. The patient's position was therefore standardised in the prone position during manometric recordings. Intra-gastric air distension has not been observed to affect SO basal pressure [348].

(2) Analysis of manometric recordings.

Four parameters were determined :(1) Duodenal pressure. This was determined from the catheter prior to cannulation. The average duodenal pressure was 8±2 mmHg. (2) Ductal pressure (CBD and pancreatic duct). These were usually 5 to 15 mmHg above duodenal pressure (3) Basal SO pressure. Basal SO pressure was calculated by subtracting the duodenal pressure from the baseline SO pressure between phasic contractions in each of the channels. The SO tonic pressure was 5-8 mmHg above CBD (or 15 to 30 mmHg above duodenal pressure). (4) Amplitude, frequency, duration and direction of the phasic contractions of the sphincter. The amplitude was measured from the beginning of the slope of pressure increase from the basal pressure to the peak of the phasic contraction wave. Average phasic contractile frequency was calculated as the number of phasic contractions divided by the duration of the recording period during which contractions were identified. The duration of phasic contractions in seconds was measured from the onset of the major upstroke to the end of the wave. All measurements were expressed relative to intraduodenal pressure which was defined as zero. Ductal pressure was calculated by subtracting the duodenal pressure value from the pressure recorded in each of the orifices. The amplitude of SO pressure was calculated as the average of maximal contractile amplitude minus basal pressure in each of the three channels.

A motility index (MI= Amplitude x Frequency) was calculated as the product of the SO mean phasic amplitude and frequency during the period of the recording [349].

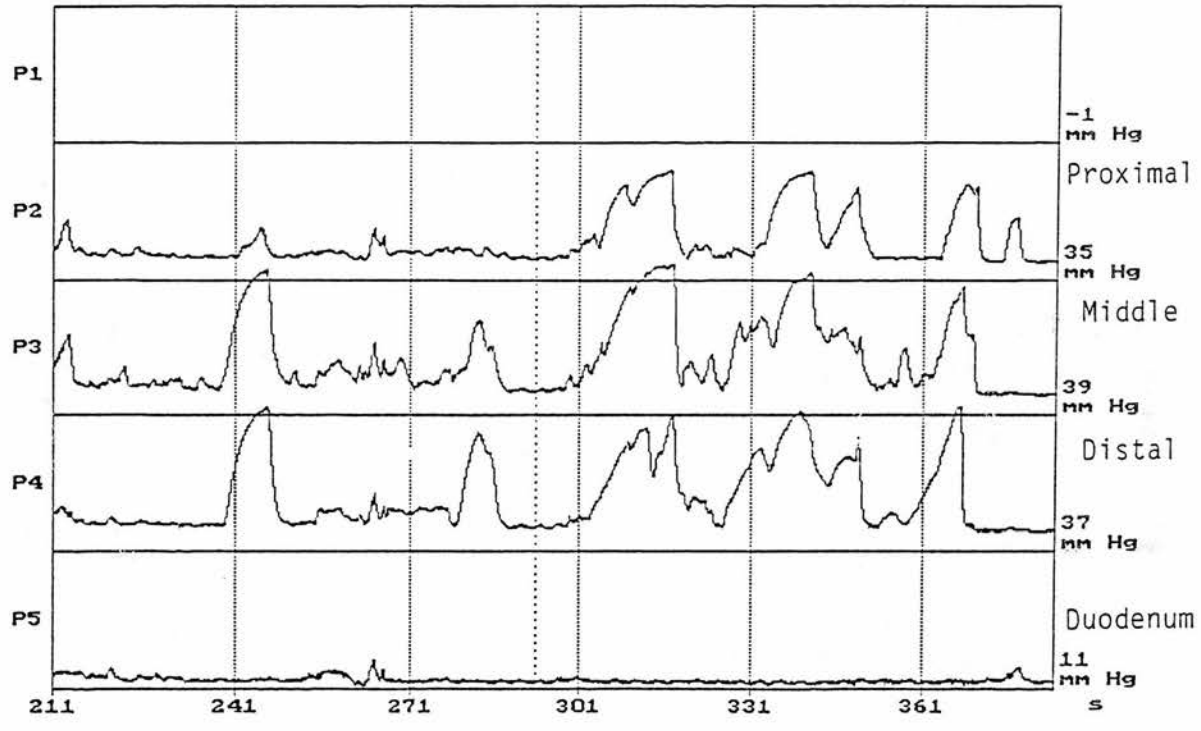


Figure 5.3 Manometric tracing of SO motility at paper speed of 4 cm/min (the top three tracings represent proximal i.e. most cranial, middle and distal sphincter pressure; the bottom tracing shows the duodenal pressure).

Figure 5.3 is an example of a typical manometric tracing. Several manometric characteristics have been identified as criteria for SO dysfunction (table 5.3). These findings have been independently validated by different investigators [65,66,346,350-355].

Table 5.4 Criteria for abnormal manometry

Basal pressure >40 mmHg
Peak pressure >240mmHg
Paradoxical response to CCK
Frequency of phasic wave contractions>7
Retrograde contractions >50%

Elevated SO basal pressure is the most common abnormality. Endoscopic sphincterotomy has been known to decrease the pressure with successful alleviation of symptoms [350,352]. In normal subjects, intravenously administered CCK decreases SO pressure and abolishes phasic waves. In some patients with SO dysfunction, CCK causes a paradoxical increase in basal pressure [354,355]. An increase in phasic wave contraction greater than 7 per minute is considered to be abnormal. This is a rarely observed finding in patients with SO dysfunction and is described as tachyoddia [356]. Phasic wave contractions are propagated antegradely in normal subjects. Retrograde phasic wave has been described but is rarely seen in patients with SO dysfunction [353].

Intrinsic variability of SO motor function has been evaluated by comparing two perendoscopic SO manometric recordings performed in the same session or in different sessions several days apart [346,357,358]. No significant difference in the manometric variables was found between the intra-session and inter-session measurements. Intra-session measurements showed a high correlation for SO basal pressure, SO length, the amplitude and frequency of phasic activity. Inter-session measurements showed a high correlation for SO basal pressure and SO length and a low correlation for amplitude, duration and frequency of phasic activity. It therefore

appears that biologic time related variation of SO motor function affects phasic contraction though resting basal SO pressure is unaffected. This is perhaps expected as phasic contractions is affected by interdigestive motor cycles. Interestingly, one study on healthy volunteers shows excellent reproducibility [346]. Perhaps the inter-session variability in the phasic contractions were noted in studies of patients with suspected SO dysfunction and is due to the episodic nature of the disorder.

(3) Factors influencing interpretation of manometric findings

Several artifacts may be present. Intra-abdominal pressure variations due to retching, coughing, deep breathing may be transmitted to the recording sensor. Variation in the spatial relationship between the catheter and the papilla occurs during respiratory effort, retching and body movement. Displacement of the catheter may also occur during manometry. It is important for the manometrist to mark on the recording all these events that interfere with interpretation of tracing. Another manometric catheter probe is taped on the external surface of the endoscope which helps to determine occurrence of duodenal contractions and retching (see bottom tracing on figure 5.3). Contractile activity which occurs simultaneously with duodenal contraction is ignored as it is felt to be secondary to duodenal contraction. An artificial elevation of the pressure may be noted if the catheter tip is impacted against the ductal wall. Defects in the perfusion system such as air bubbles, leakage or occlusion of the catheter may reduce the sensitivity of the system.

The choledochal and pancreatic sphincters are distinct physiological entities, corresponding to the anatomical structures described by Boyden. Evidence supporting the pancreatic duct as a distinct physiological entity came from manometric studies after endoscopic sphincterotomy [358-360]. The choledochal sphincter was abolished following sphincterotomy while the pancreatic sphincter continued to demonstrate normal motility. However, there was minimal increase in the basal pressure and decrease in the amplitude of the pancreatic sphincter compared with the choledochal readings [346,347]. A proportion of patients (42%) with partial CBD obstruction were found to have higher basal sphincter pressures in the

choledochal sphincter alone or both of the sphincters. Similarly, 29% of patients with acute recurrent pancreatitis had elevated pancreatic sphincter pressure alone or in combination with choledochal sphincter. It therefore seems that the two sphincters are functionally independent but yet related in their motor function as there do not seem to be significant differences in the values of their motor activity.

(4) Complications

SO manometry is associated with cholangitis and pancreatitis. The overall incidence of pancreatitis was estimated at 5% [361]. The rate was higher in patients with pre-existing pancreatitis. Twenty eight percent of patients with chronic pancreatitis developed an exacerbation of their pancreatitis.

The aetiology of post-sphincter of Oddi manometry pancreatitis is unknown but probably multifactorial. Mechanical trauma from cannulation, hypotonic injury from sterile water, and hydrostatic injury from overfilling of pancreatic duct have all been implicated [362]. Repeated attempts at cannulation can result in oedema and subsequent occlusion of the papilla. During ERCP, the pancreatic ductal system typically accepts 1 to 3 ml of contrast before acinarisation occurs. Therefore a triple-lumen catheter that delivers 0.75 ml of perfusate per minute causes overfilling and acinarisation after 1 to 2 minute. Bacterial cholangitis and pancreatitis should be a rare occurrence with high grade disinfection and pre-procedure administration of antibiotics.

Pancreatitis is very uncommon if only the choledochal sphincter is studied. When manometry is performed in the pancreatic duct, repeated cannulation and prolonged stationing of the catheter in the duct should be avoided to prevent pancreatitis. A specially designed catheter that allows continuous aspiration of fluid from one of the three lumen can maintain the pancreatic duct pressure at a low level. This catheter has been shown to reduce the incidence of pancreatitis (23.5% versus 3%) although this has only been reported from one centre [362].

5.3.2 Microtransducer technique

A catheter with a miniaturised intraluminal transducer, 4 Fr in diameter, located at the distal tip has been used by Japanese investigators to measure intraductal pressure [344]. This system, which avoids the infusion of fluid, allows intrabiliary pressure to be recorded for prolonged period of time and theoretically, avoids the risk of pancreatitis due to over distension of pancreatic duct. No study has compared this technique with the perfusion systems. The transducers are costly and subject to baseline drift. They are not suitable for measuring SO pressure due to the difficulty of keeping the sensor within the sphincter. The position of the catheter in the SO could be maintained with the use of a guide wire that is passed into the centre of the catheter. However, fluoroscopy is needed to check the position of the radiopaque sensors in relation to the guide wire and duodenal wall [348]. This technique looks promising and offers the prospect of prolonged telemetric recording provided a technique can be developed to station the transducer in the SO.

5.4 Isometric tension measurements of muscle strip in tissue baths

In vitro water bath experiments with isolated tissues allow direct examination of muscle contractility with different reagents. The main stumbling block with experiments of this nature was retrieval of viable human tissue from LC. Bovine gallbladders were therefore studied.

5.4.1 Tissue preparation

Bovine gallbladders obtained from a local abattoir were used for the experiments. Tissue was obtained half an hour to an hour after death of animals. Whole gallbladders were removed and transported in flasks containing cooled (4^0C), pre-oxygenated Kreb's solution at pH 7.4. The Kreb's solution consisted of (mM): NaCl 118, $NaHCO_3$ 25, D-dextrose 11, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2 and KH_2PO_4 1.2. The flasks were kept cool in an ice box. Pieces of gallbladder (about 10 cm long and 10 cm wide) were placed in a Petrie dish under cooled oxygenated Kreb's solution and four muscle strips (10 mm long and 2-3 mm wide) were obtained from each gallbladder. The orientation of muscle strips is not important as muscle fibres are loosely arranged in longitudinal,

circular and oblique bundles [1]. Experiments were started within one hour after removal. We have found bovine gallbladders to remain viable up to four hours after removal. Strings were tied into knots at both ends and each strip was mounted for isometric tension recording in four 10ml organ baths containing Kreb's solution at 37 ^{o}C, continuously bubbled with O_2:CO_2 (95%:5%).

Muscle strips were equilibrated at basal tension 1g for at least 1 hour before experiments. Kreb's solution in the water baths was changed every 15 minutes. Four water baths could be set up simultaneously.

5.4.2 Equipment

One end of the muscle strips was connected to an isometric transducer and chart recorder. Changes in tension were monitored by isometric force transducers connected to a MacLab Analog Digital Instruments and a personal computer with MacLab Chart V3.2. (see figure 5.4). The polygraphs on the video screen show contractility of the muscle strips in the four water baths simultaneously. For each graph, an area over time could be "selected" and the computational software could then calculate the mean tension which is the average of basal tension and peak of phasic contraction over the specified time period. One minute was chosen as the standard time period (see figure 5.5 for example of the polygraph).

5.4.3 Preparation of chemicals

1. Preparation of L-NMMA

Raw materials :

1. L-N^G-monomethyl-arginine (L-NMMA, Calbiochem-Novabiochem, UK). Molecular weight is 248.
2. 0.9% sodium chloride injection B.P. (Baxter Healthcare Ltd, Thetford, Norfrolk, UK)

Preparation :

1. L-NMMA was dissolved with normal saline to a dilution of 1mg/ml ($4x10^{-3}$ M).

2. Stock solutions of 2 and $4x10^{-3}$ M were then stored in plastic or glass containers at -20^0C and used within a week of preparation.

2. Preparation of cholecystokinin

Raw material :

1. Cholecystokinin (CCK) (Ferring Pharmaceuticals, Malmo, Sweden)

This preparation of CCK contains equimolar amounts of CCK33 (molecular weight 3663) and CCK39 (molecular weight 4541). The average molecular weight of the preparation is thus 4102. 1mg of the preparation contains approximately 1000 IDU. Therefore 1 IDU is 0.001mg in weight.

Preparation :

1. Three different stock solutions of cholecystokinin was prepared as follows:
2. An ampoule of CCK containing 70 IDU was dissolved into 70ml of Kreb's solution.
3. From the molecular weight of CCK, therefore 1 IDU/ml was equivalent to ($0.001/4102=2.4x10^{-7}$)M.
4. Three different stock concentrations of 10^{-7}, 10^{-8}, 10^{-9} were made.
5. These stock solutions were placed into glass or plastic containers and stored deep frozen (-20^0C) for no more than one week.
5. The solutions were thawed in a refrigerator before use.

3. Preparation of glyceryltrinitrate

Raw material:

1. Glyceryltrinitrate (Nitrocine, Schwarz Pharma, UK). Molecular weight is 227.1

Preparation:

Glyceryltrinitrate was purchased in 10mg ampoules each at concentration of 1mg/ml. Glyceryltrinitrate was used at this concentration that was approximately $5x10^{-3}$M.

4. Preparation of sodium nitroprusside

Materials :

1. Sodium nitroprusside (Sigma, St Louis, USA). Molecular weight was 298.

Preparation :

Stock solution of 10^{-1}M is made by dissolving 21mg of sodium nitroprusside in 1ml of solution. The stock solution is prepared fresh before use and the glass container is immediately covered with aluminium foil.

5. Preparation of potassium chloride (KCl)

200µl of 1M stock solutions, when diluted in 10ml of Kreb's solution, would achieve KCl concentration of 20mM

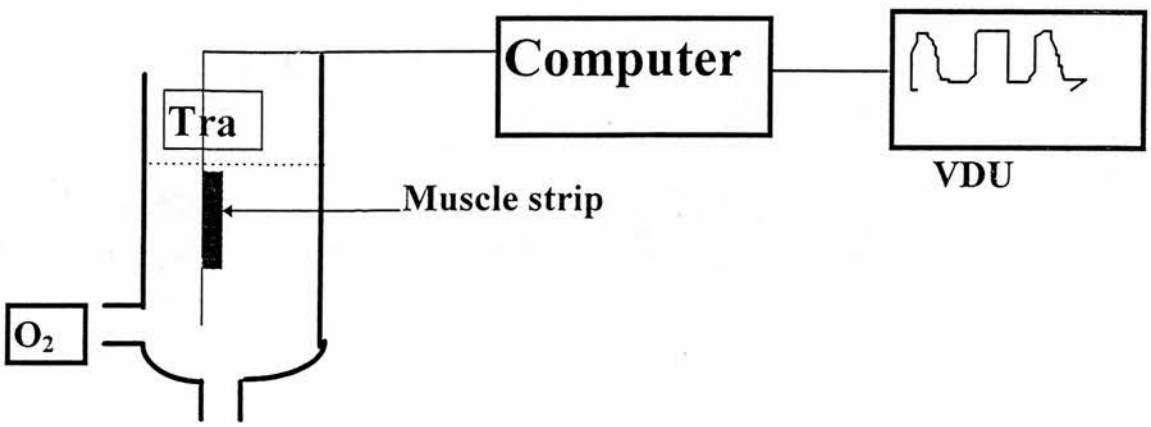


Figure 5.4 Measurement of muscle contraction.

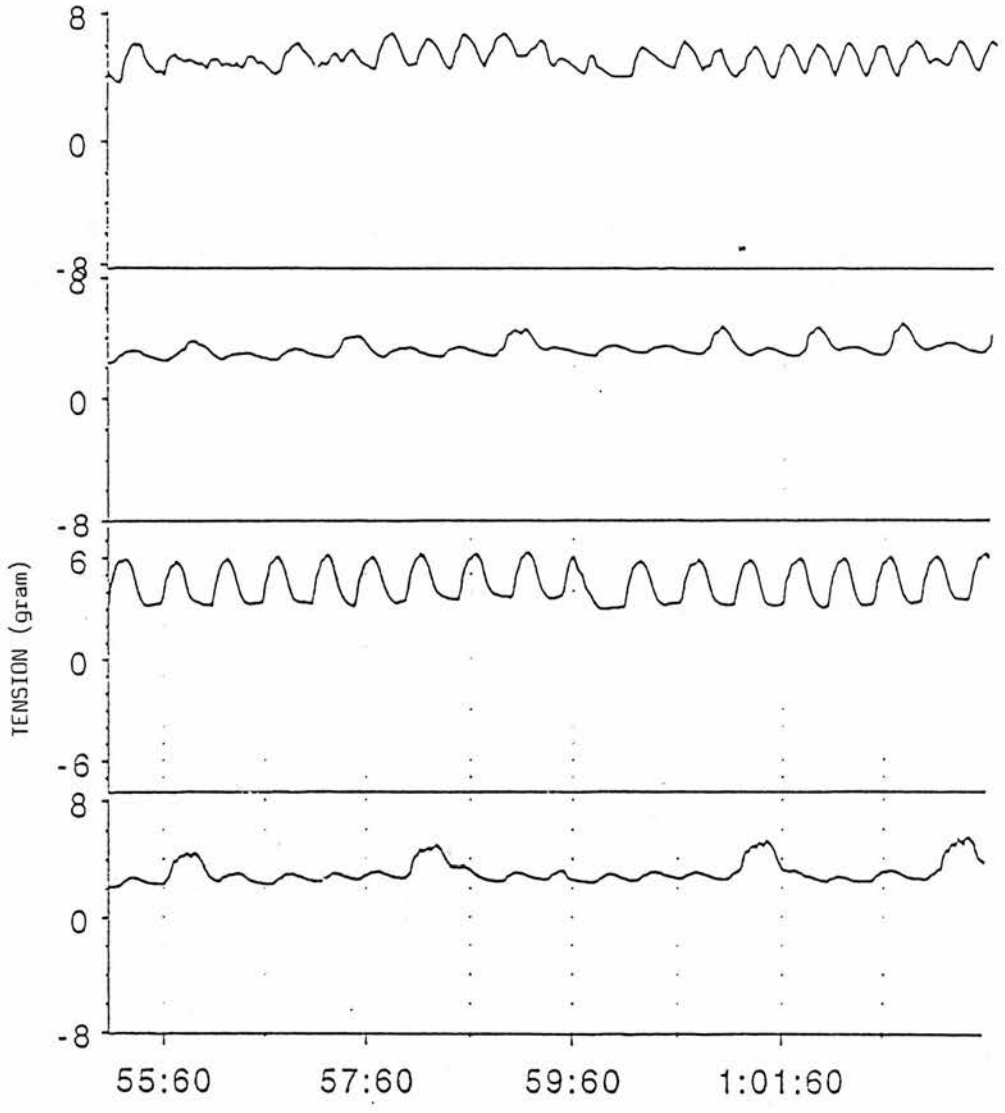


Figure 5.5 Typical polygraph showing contractility of muscle strips. The y-axis represents tension in gram; each graph shows muscle strip contractility in each water bath. The pattern of muscle contractility is different although all the strips were taken from adjacent areas of gallbladder.

5.4.4 Dose response curve for KCl

(1) Method

The dose response to KCl was examined at cumulative bath concentrations of 20 mM-100 mM. After one hour of equilibration, muscle strips were stimulated to contract with KCl. At each concentration of KCl, five minutes was allowed for muscle strips to achieve maximum contraction before the tension was noted. Four strips from each of six gallbladders were examined (n=24).

(2) Results

Some muscle strips showed spontaneous basal contraction with superimposed phasic contractions. KCl achieved immediate contraction with increase of tone and stimulation of phasic activity. The dose response curve of KCl is shown in figure 5.6. The maximum increase in tension was 3.5g. From the curve, the effective concentration at 80% (EC_{80}) is calculated to be 50 mM.

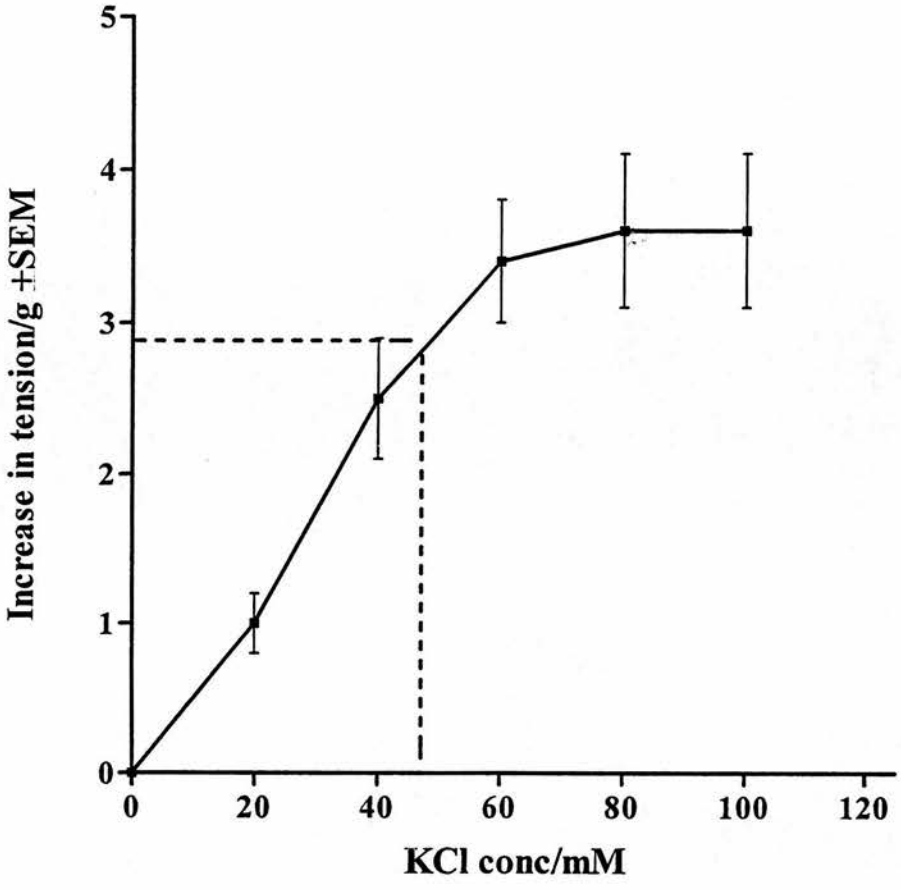


Figure 5.6 Dose response curve for potassium chloride.

5.4.5 Maximal basal tension in response to EC_{80} KCl concentration

The aim of this experiment was to determine the basal tension that achieved maximum contraction in response to 50 mM KCl.

(1) Method

Four muscle strips were obtained from eight gallbladders. Muscle strips were each equilibrated at a basal tension ranging from 1 to 4g for an hour. They were then stimulated to contract with 50 mM KCl. The maximum tension reached after five minutes was recorded. The mean increase in tension at each basal tension was calculated.

(2) Results

Some of the muscle strips had spontaneous tonic and phasic activity. Consequently, the actual basal tension after the equilibration period was sometimes higher than the designated basal tension for those muscle strips adjusted at a basal tension of 1 g. The tension increased from 1 to 1.6 ± 0.3 g. There was progressive increase in contractility as the basal tension was increased from 1 g to 3 g; the degree of contraction became suboptimal as the basal tension was increased to 4 g (see figure 5.7). Basal tension of 2 g and 3 g produced significantly higher degree of contraction (ANOVA). However, the change in tension between basal tension of 2 g and 3 g was not significantly different (t-test); therefore 2 g basal tension was chosen as the basal tension for subsequent experiments.

Initial designated basal tension (g)	Basal tension after equilibration±SE (g)	Tension after KCl± SE (g)	Change of tension±SE (g)
1	1.6±0.3	3.2±0.4	2.2±0.4
2	2.1±0.2	5.1±0.4	3.3±0.4
3	3.1±0.1	6.6±0.5	3.5±0.5
4	4.1±0.1	5.3±0.3	1.1±0.4

Table 5.4 Mean tension of muscle strips before and after stimulation with 50mM KCl at varying basal tension.

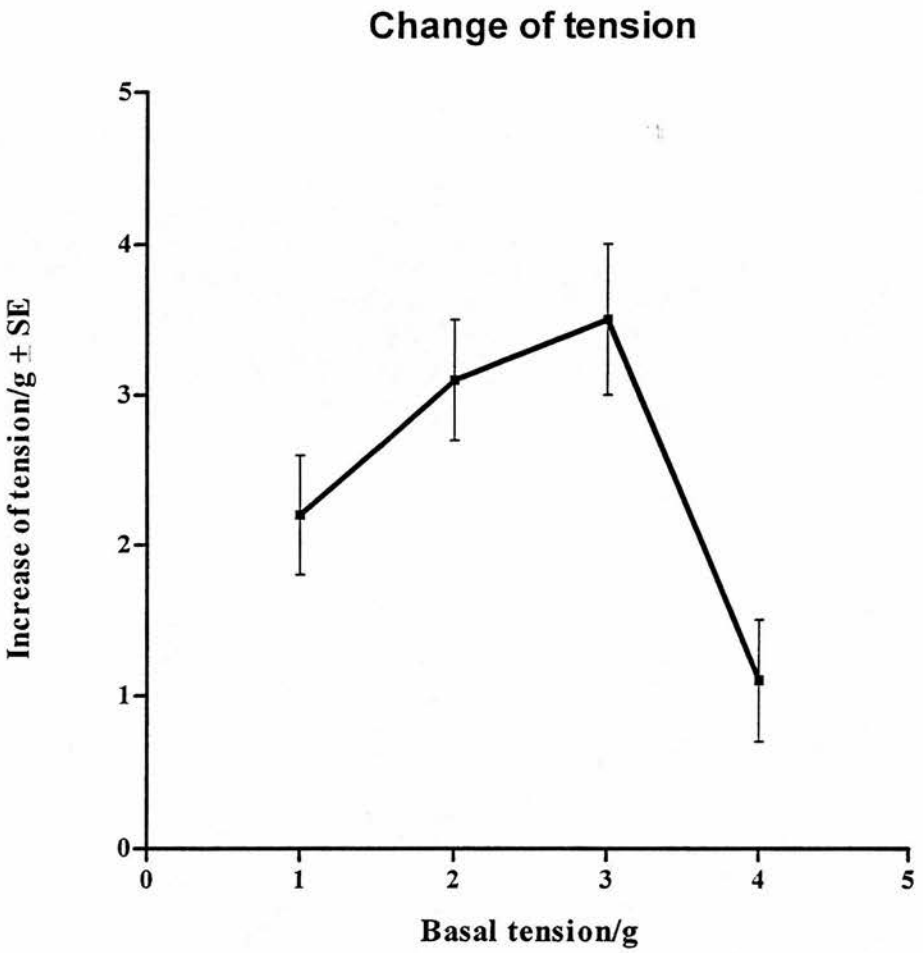


Figure 5.7 Increase of tension after stimulation with 50 mM KCl at varying basal tension.

5.4.6 Dose response curve for CCK

(1) Method

The final part of this initial experiment was to determine the 50% effective concentration (EC_{50}) for CCK. Muscle strips were equilibrated for an hour at basal tension of 2g. Five gallbladders were examined at cumulative bath concentrations of CCK between 10^{-11} to $1.0x10^{-9}$ M; five further gallbladders were subsequently examined at cumulative concentration between $5.0x10^{-9}$ M to $1.8x10^{-8}$M. Five minutes intervals were allowed at each concentration before addition of further stock CCK solutions.

(2) Results

Maximum contraction was achieved at CCK concentration of $1.2x10^{-8}$ M. EC_{50} for CCK is found to be at $5x10^{-9}$M (figure 5.8).

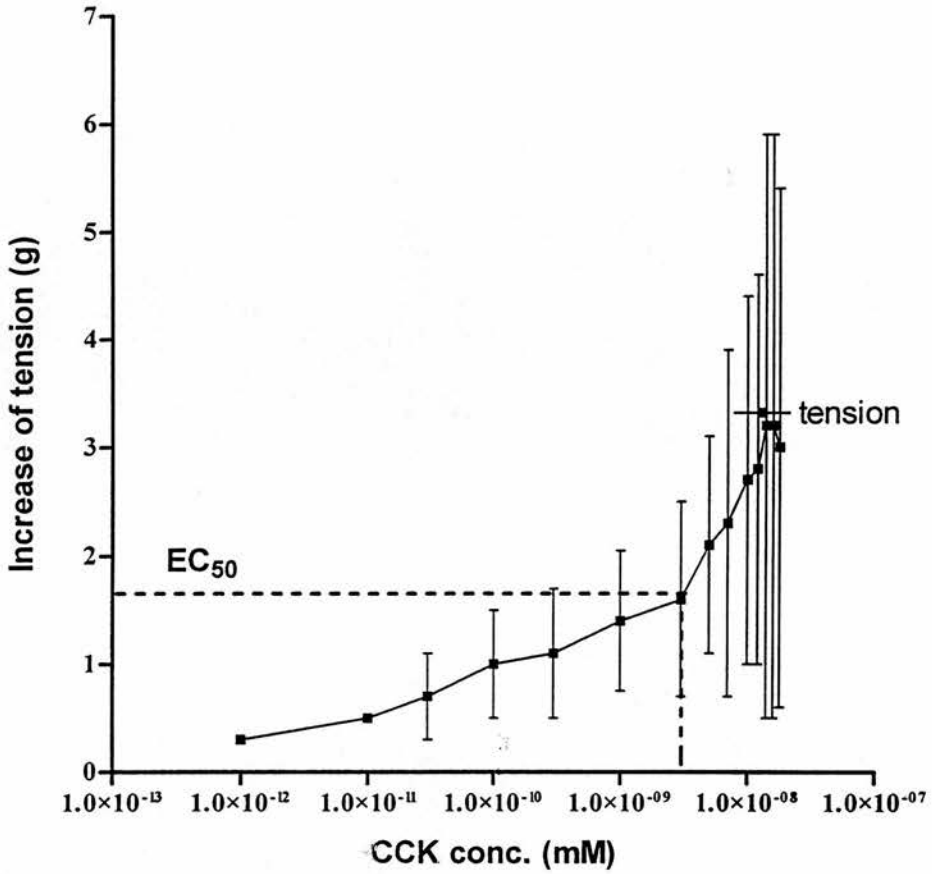


Figure 5.8 Cumulative dose response for CCK.

5.5 Radioimmunoassay

Blood samples were obtained from an intravenous catheter and were kept on ice; plasma was separated within 30 minutes and stored at -70^{0}C for subsequent assay of cholecystokinin (CCK) and gastrin. Plasma CCK and gastrin were measured by Drs. Joy Ardill and Lee Armstrong at Department of Medicine, Queen's University, Belfast.

5.5.1 Cholecystokinin

Plasma CCK concentrations were measured using a highly sensitive and specific radioimmunoassay. The antiserum R7(3) used, binds to all carboxy-terminal CCK peptides containing the sulphated tyrosine. Relative cross reactivity with CCK8 is 1.0, CCK33 is 0.72, CCK39 is 0.7. Cross reaction with sulphated gastrins is less than 1% and no cross reaction is observed with other gastrointestinal peptides. Synthetic CCK8 labelled ^{125}I using Bolton and Hunter Reagent (Amersham, England) was used as tracer and synthetic sulphated CCK8 (Bachem, England) was used as standard. Separation of antibody-bound from free peptide was achieved using dextran coated microfine charcoal. The detection limit of the assay was 1 ng/L (1 pmol/L) and intraassay and interassay variation was 8% and 11% respectively at 5 ng/L.

5.5.2 Gastrin

Plasma gastrin was measured by radioimmunoassay as previously described by Ardill [363]. All gastrin measurements were performed using a C-terminal reactive antiserum (R98) which measures only amidated gastrins. R98 detects G17 and G34 in equimolar concentration and measures both sulphated and unsulphated variants. Cross reactivity with CCK is <0.001%. The assay as used in this study had a detection limit of 2ng/L (1pmol/L) and intra and interassay variation was 4.6 and 7.4% respectively at 25 ng/L. This antibody does not react with pentagastrin.

5.6 Gastric emptying

5.6.1 Methods

Gastric emptying of a radio-labelled omelette was measured. Omelettes were radiolabelled with 4mBq of Tc ^{99m} sulphur colloid as described by Millar et al [364]. Previous validation method had shown that less than 1% of the radiolabel became separated from the test meal during incubation with gastric acid [364]. Subjects were positioned in front of a gamma camera counter and anterior and posterior scans were obtained after the omelette had been swallowed. An area of interest over the stomach was identified and pairs of frames were obtained at 2 minute intervals. The median of anterior and posterior radioactivity were calculated. Gastric emptying was calculated as described by Millar et al using a computer which plotted best fit for the data.

5.7 Immunohistochemistry of NOS

Tissue specimens were prepared for immunohistochemistry by standard methods. Paraffin embedded sections were washed with xylene and alcohol. Sections were rehydrated in distilled water for five minutes and endogenous peroxidase activity were removed with 2% H_2O_2 in 60% methanol for thirty minutes. Slides were covered with normal sheep serum in phosphate buffered saline (PBS) and incubated overnight with neuronal specific NOS antiserum. Antiserum to neuronal specific NOS was purchased from Affiniti Research Products Limited, Exeter, UK. The synthetically manufactured peptides corresponded to amino acid residues 1414-1429 of the rat neuronal NOS-1 protein [283] modified to facilitate specific N-terminal conjugation to a carrier protein. The primary antibody was applied at dilution between 1/200 to 1/1000 in Tris buffered saline (TBS) containing 1% bovine serum albumin. Sections were incubated for 1 hour at room temperature with the primary antibodies. Sections were washed in TBS for 10 minutes and successively incubated with secondary antiserum, biotinylated anti rabbit antiserum and streptavidine reagent. Peroxidase activity was revealed using diaminobenzidine with copper enhancement. Sections were counterstained, dehydrated, and mounted in DPX (distrene, tricrecyl phosphate, xylene). For identification of neurons, antibodies against marker proteins for neural perikarya, PGP 9.5 (protein gene product) was applied. PGP 9.5 is a ubiquitin carboxy terminal peptidase which is abundantly expressed by both peripheral and central nervous fibres and is present within the

axoplasm. It is an excellent marker for axons, including small unmyelinated nerve fibres and showed up innervation of gallbladder well on immunohistochemistry [365].

PART TWO

EXPERIMENTAL WORK

CHAPTER SIX

EARLY GALLBLADDER EMPTYING

6.1 Introduction

As stated in section 4.1, mechanisms which may be responsible for the early phase of gallbladder emptying were examined. Early gallbladder emptying could be mediated neurally in response to the anticipation of food; that is, there may be a cephalic phase of gallbladder contraction effected by the vagus nerve. Alternatively, gastric distension might stimulate release of hormones which act upon gastrointestinal motility. Thus plasma CCK and gastrin concentration were measured in relation to gallbladder and gastric emptying in a range of experimental situations.

6.2 Subjects and Methods

Two experiments were performed. The first examined the relationship between gastric emptying and gallbladder contraction. The second examined putative mechanisms of the early phase of gallbladder emptying.

6.2.1 Simultaneous measurement of gastric and gallbladder emptying

Seven subjects (5 males, aged 40-55 years) were recruited from medical staff. None had a history of dyspepsia, peptic ulcer, gallstones, diabetes or thyroid disease. All had a body mass index of less than 30 and none smoked. All volunteers tested negative for *Helicobacter pylori* by the C^{14} Urea Breath test.

Gallbladder and gastric emptying were measured simultaneously following ingestion of a test meal comprising a two eggs omelette (10.8g of fat and 147 kcal), eaten within 3 minutes. The omelette was labelled with 15 mBq of Tc^{99m} before cooking and solid gastric emptying was measured as described in section 5.6. Radioactivity over the area of interest was measured at 10-minute intervals for 90 minutes. Gallbladder volumes were measured by ultrasound scans at similar intervals following the meal.

6.2.2. Early gallbladder emptying

This was an open, within subject study involving a further group of 8 healthy non-smoking male volunteers (age 27-37 years) recruited from medical and laboratory staff.

(1) Measurement of gallbladder emptying

Each volunteer was studied after an overnight fast on three separate mornings in random order : (1) *Fatty meal* The test meal comprising a two egg omelette was eaten within 3 minutes. (2) *Modified sham feeding* Volunteers were asked to chew and then spit an omelette into a receptacle for a 3-min period. They were instructed not to swallow the food and to rinse the mouth with water between each morsel. (3) *Gaseous distension* Gallbladder volumes were measured during gastric distension caused by ingestion of two sachets of effervescent powder (Carbex Granules, Ferring Pharmaceuticals, U.K.). This compound is routinely used in double contrast barium meal and one sachet is known to produce 250 ml of carbon dioxide.

During each phase of the study, gallbladder volumes were measured by ultrasound scan. Measurements were taken at 10 minutes intervals for 30 minutes prior to ingestion of food or effervescent powder. Subsequent measurements were taken at 5 minute intervals for 30 minutes, at 10 minutes intervals for a further 30 minutes and at 15 minute intervals for the last 30 minutes.

(2) Measurement of gut hormones

Blood samples were obtained from an intravenous catheter at the same time intervals as ultrasound scan measurements. The samples were kept on ice; plasma was separated within 30 minute and stored at -70^0C for subsequent assay of CCK and gastrin. Plasma CCK and gastrin concentrations were measured as described in section 5.5.

(3) Statistical analysis

Measurements of gallbladder volumes and gut hormones were performed in duplicates, and the means were noted. Gallbladder volume was expressed in millilitre and as a percentage of fasting volume. Ejection fraction (EF) was calculated by the formula :

[(fasting volume-gallbladder volume)/fasting volume x 100].

Integrated CCK and gastrin concentrations for each individual volunteer were determined by calculating the area under the CCK and gastrin concentration-time curves after subtraction of basal values. Results for the group are expressed as mean±SEM. Statistical analysis was performed by analysis of variance. Differences with a two-tailed $p<0.05$ were considered significant.

The studies were approved by the Lothian Ethical Subcommittee for Medicine and Oncology.

6.3 Results

6.3.1 Simultaneous gastric and gallbladder emptying

Mean serial postprandial gallbladder volumes and gastric emptying are depicted in figure 6.1. Gallbladder emptying started immediately after ingesting the test meal and preceded gastric emptying. Significant emptying of solids from the stomach started after a mean lag period of 30±10 minutes. By this time, the gallbladder had emptied by 35.7±14%

6.3.2 Gallbladder emptying : early phase

There was no significant difference in mean fasting gallbladder volume ± SEM on the three different days. That for test meal alone was 22.9 ± 2.7 ml, sham feeding was 22.7 ± 3.6 ml and gaseous distension was 21.7 ± 4.4 ml..

(1) Test meal

Fatty meal ingestion was followed by immediate gallbladder emptying at a mean rate of 0.57ml/min, with an ejection fraction (EF) of 25% by 10 minutes (figure 6.2). Only two subjects had an EF of less than 10% in the first 10 minutes. The initial phase of gallbladder emptying was associated with increases in mean immunoreactive plasma CCK concentrations from 6.2±0.9 ng/L to 10.1±3.2 ng/L. Mean plasma gastrin concentrations also increased following the meal, from 26.3±4.7 to 31.5±6.2 ng/L (figure 6.3).

After a mean period of 25 minutes, the rate of gallbladder emptying slowed to 0.19±0.05 ml/min and this was associated with a second, more sustained rise of plasma CCK concentrations to 9.5±1.5 ng/L and of gastrin to 34±5.4 ng/L. Mean integrated CCK and gastrin concentrations were 237±70 ng/L and 974±297 ng/L respectively (figure 6.5).

(2) Modified Sham Feeding

Gallbladder emptying did not occur in six of the subjects after the sham feed, and the mean gallbladder volume of the whole group was unchanged by sham feeding (figure 6.2). Plasma CCK and gastrin concentrations remained at basal levels (figure 6.4). However, in two subjects sham feeding stimulated gallbladder emptying with an EF of at least 25% at 20 minutes in both cases (figure 6.6 and 6.7). In these subjects CCK concentrations increased from 13 to 18 ng/L, but plasma gastrin concentrations did not change (figure 6.6). Integrated CCK and gastrin concentrations for the whole group were 39±13 ng/L and 59.6±31 ng/L respectively. The integrated concentrations of CCK and gastrin were significantly less than the levels obtained after fatty meal (figure 6.5).

(3) Gaseous distension

No significant gallbladder emptying followed gastric distension and plasma CCK and gastrin concentrations were unaltered throughout (figure 6.2 and 6.4). There was no significant rise of integrated plasma CCK and gastrin concentrations (figure 6.5).

6.4 Discussion

This study confirmed that gallbladder emptying started before the onset of significant gastric emptying of solids. Gallbladder emptying exhibited a biphasic emptying pattern comprising a rapid early phase followed by a slower phase [366]. The early phase of gallbladder emptying was associated with increases in immunoreactive plasma CCK and gastrin concentrations. In the majority of individuals modified sham feeding did not provoke gallbladder emptying, but in two this did occur with EF of more than 25% at twenty minutes and emptying was associated with increases of plasma CCK

concentration. Gaseous gastric distension was not associated with gallbladder emptying, nor with changes in plasma CCK and gastrin concentrations.

The stimulus for increased plasma immunoreactive CCK concentrations noted immediately after eating is unknown, but it is reasonable to speculate that release of CCK is responsible for the early phase of gallbladder contractions. The plasma concentration achieved immediately after eating was above 10 ng/L, consistent with the threshold plasma level of 1.5 pM previuolsy identified for gallbladder contraction [398]. It is unlikely that entry of a proportion of the fatty meal into the duodenum stimulates early release because the time course of gallbladder contraction is so rapid. Equally, gastric distension by the test meal seems an unlikely stimulus for hormone release and gallbladder contraction because the volume of the test meal was modest and because gaseous distension failed to cause the gallbladder to empty or hormone concentrations to rise. Gastrin is unlikely to be responsible for gallbladder contraction. It causes gallbladder muscle contraction in some species, though much less potently than CCK [19] and not at all in man [20].

We postulate several mechanisms for the early phase of gallbladder emptying and release of CCK. This is illustrated diagrammatically in figure 6.8. There is intramural plexus in gallbladders that contain cholinergic neurons supplied by the anterior branch of vagus nerve [13]. During the cephalic phase, it is possible that gallbladder contraction could be stimulated by vagal cholinergic stimulation directly on gallbladder smooth muscle. It is known that direct cholinergic stimulation by bethanechol produced significant gallbladder emptying and cholinergic blockade with atropine diminished the gallbladder emptying responses to food and CCK [27,28]. This effect of cholinergic blockade appeared to be mediated by a selective muscarinic M_2 receptor [29]. It is also possible that vagal mechanism during the cephalic phase can stimulate the release of CCK. CCK neurons are found in the myenteric and submucous plexus, and circular muscle layers of the small bowel and colon [367]. CCK neurons are abundant in the coeliac plexus and vagus nerve [368].

Like others an inconsistent cephalic phase of gallbladder emptying in response to sham feeding was observed between individuals [333,334,369]. This may be due to vagal cholinergic neural mechanisms as cholinergic blockade with atropine inhibits gallbladder emptying in response to sham meals [333,370]. Fisher et al [333] and Hansen et al [334] also showed that atropine inhibited the cephalic phase of gallbladder emptying although they did not measure CCK concentration during their studies. Whilst most subjects did not exhibit gallbladder contraction following sham feeding, this did occur in two of the eight volunteers. In these two subjects, plasma CCK but not gastrin concentrations increased in association with gallbladder emptying. This could be due to subject variation in the cephalic stimulation of CCK secretion as has been noted for pancreatic polypeptide release after sham feeding [371]. It is unlikely that the rise in CCK was due to inadvertent swallowing of food as the duration of sham feeding in this study was only three minutes and there was no rise of gastrin concentration in these two individuals to suggest entry of nutrients in the stomach.

It is possible that CCK is released in response to entry of nutrients into the stomach i.e. gastric phase. Indeed gastropancreatic and gastrobiliary reflexes have been described in dogs [145,372,373] and man [366,369]. This might be mediated by neural reflex sensitive to intragastric nutrients and the gallbladder smooth muscle or be due to the action of gastrointestinal hormones such as bombesin released in the stomach. Bombesin is released by intragastric nutrients immediately after eating and is known to be a stimulant for CCK release [84].

The threshold for CCK release by intraduodenal nutrient stimuli is not well documented. It is possible that small amounts of swallowed food mixed with acid fluid stimulated by gastrin, entered the duodenum causing CCK release before the detection of labelled solid particles in the intestine. This issue was not examined by the current study though Froehlich.et al in their study on a group of healthy volunteers showed that with three different isocaloric meals, pure fat meal containing 25 g triglycerides induced significantly higher CCK release compared to low fat (8 g) or dextrose meals (integrated CCK concentrations of 187±27 pM, 157±12 pM and 87±13 pM

respectively). It is therefore unlikely though not conclusively excluded, for small amounts of nutrients to flow into duodenum inducing significant CCK release as noted in this study before significant gastric emptying was detected.

The pattern of CCK release following the test meal is therefore of considerable interest (figure 6.3). Immediately after ingestion, plasma CCK concentration peaked and this was associated with the rapid, early phase of gallbladder emptying. This may be due to a combination of vagal activity (the cephalic phase) and gastric reflexes as nutrients enter the stomach and stimulate hormone release. After the initial burst of release, CCK concentration then slowly rises and plateaues, and this is associated with a second, rather slower phase of gallbladder emptying. Presumably this occurs as a consequence of food entering the upper small bowel, and stimulating CCK release.

In conclusion, the early phase of gallbladder emptying preceded gastric emptying of solid food and was associated with elevation of CCK concentrations. Cephalic vagal stimulation with modified sham feeding stimulated gallbladder emptying with CCK release in some individuals. In the majority of individuals, sham meals and gaseous gastric distension did not provoke either gallbladder emptying or CCK release. We speculate that the early phase of gallbladder emptying could be due to a combination of vagal activity and gastric reflexes in response to intragastric nutrients that stimulates CCK release and gallbladder contraction.

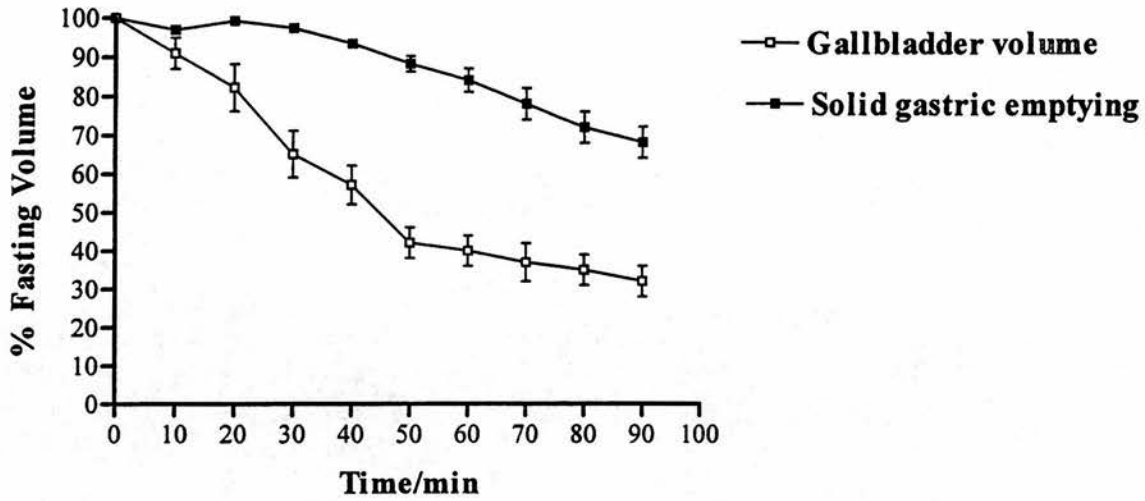


Figure 6.1 Gastric emptying and gallbladder emptying (Means+SEM) after test meal.

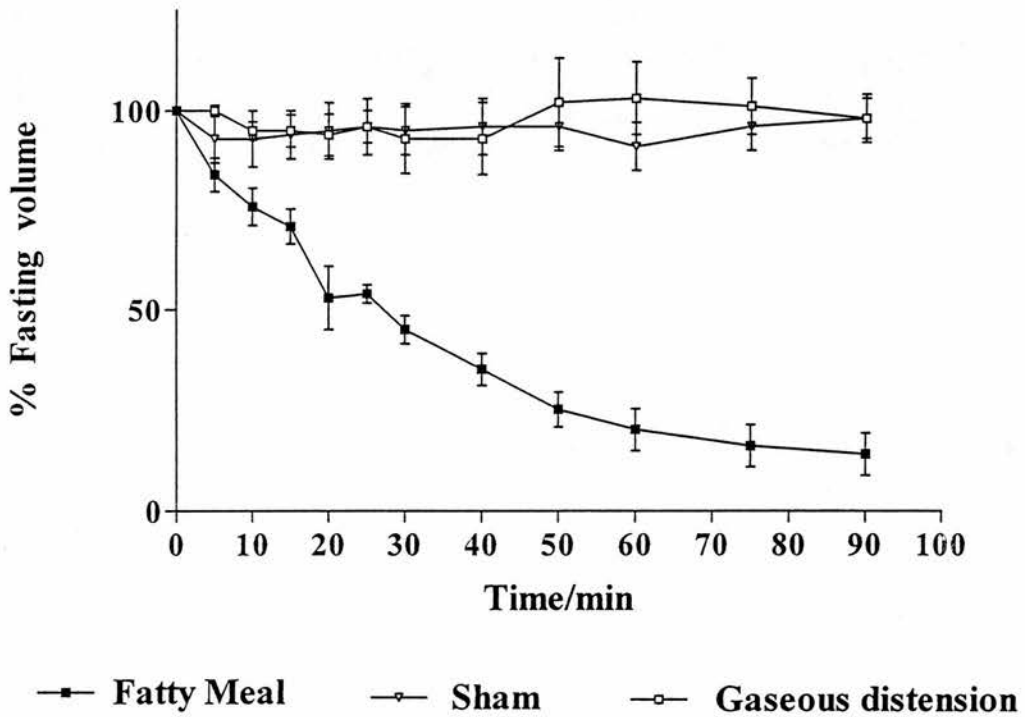


Figure 6.2. Rate of gallbladder emptying (Mean+SEM) during different test procedures.

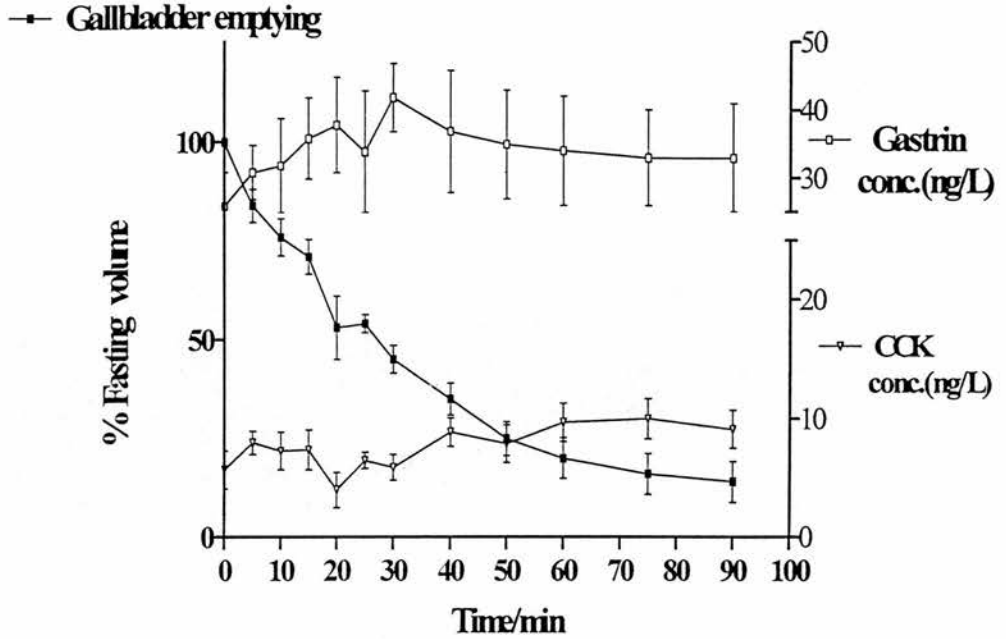


Figure 6.3. Gallbladder emptying and plasma CCK and gastrin concentrations (Means+SEM) after test meal.

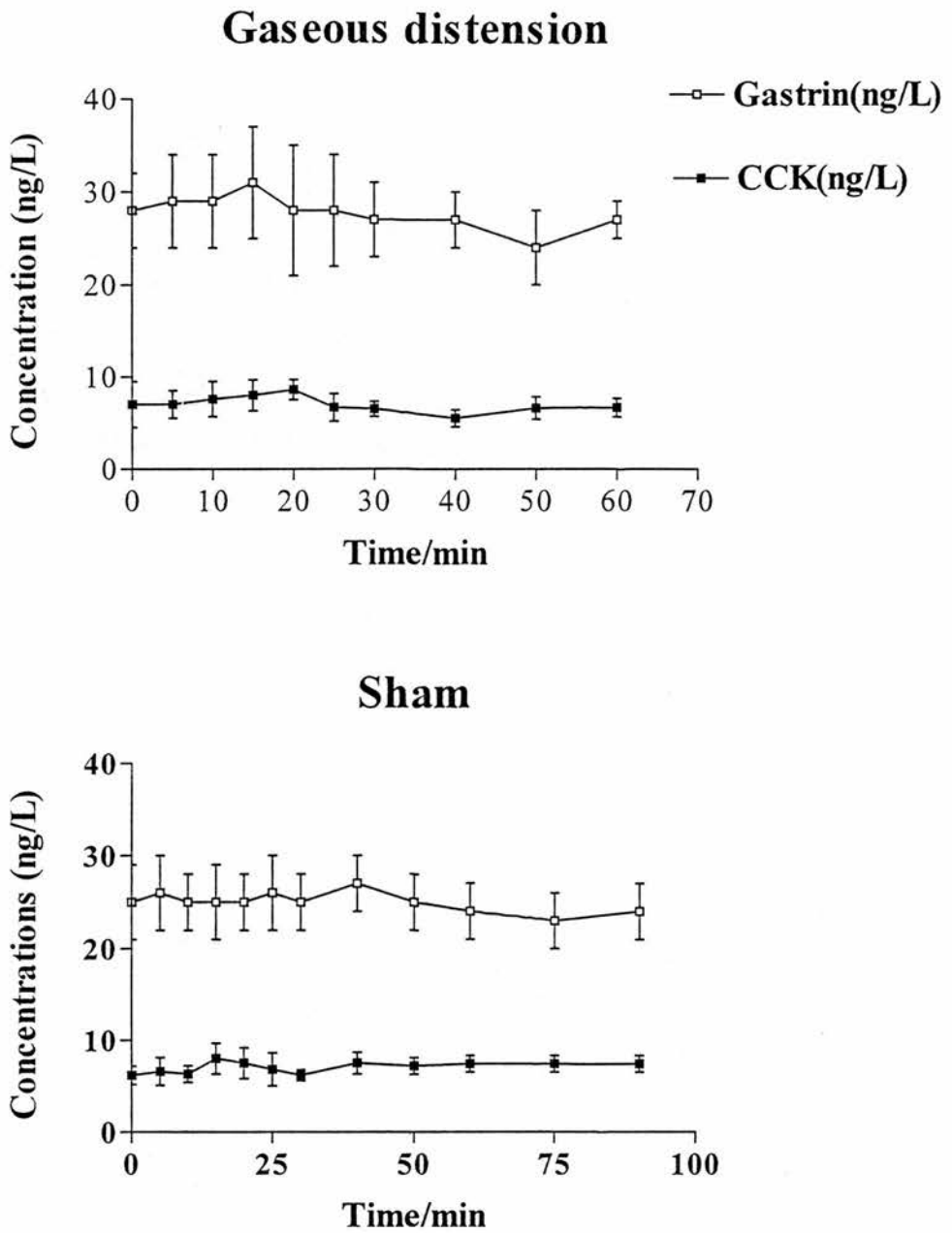


Figure 6.4. Profiles of CCK and gastrin concentrations (Means+SEM) during sham feeding and gaseous distension.

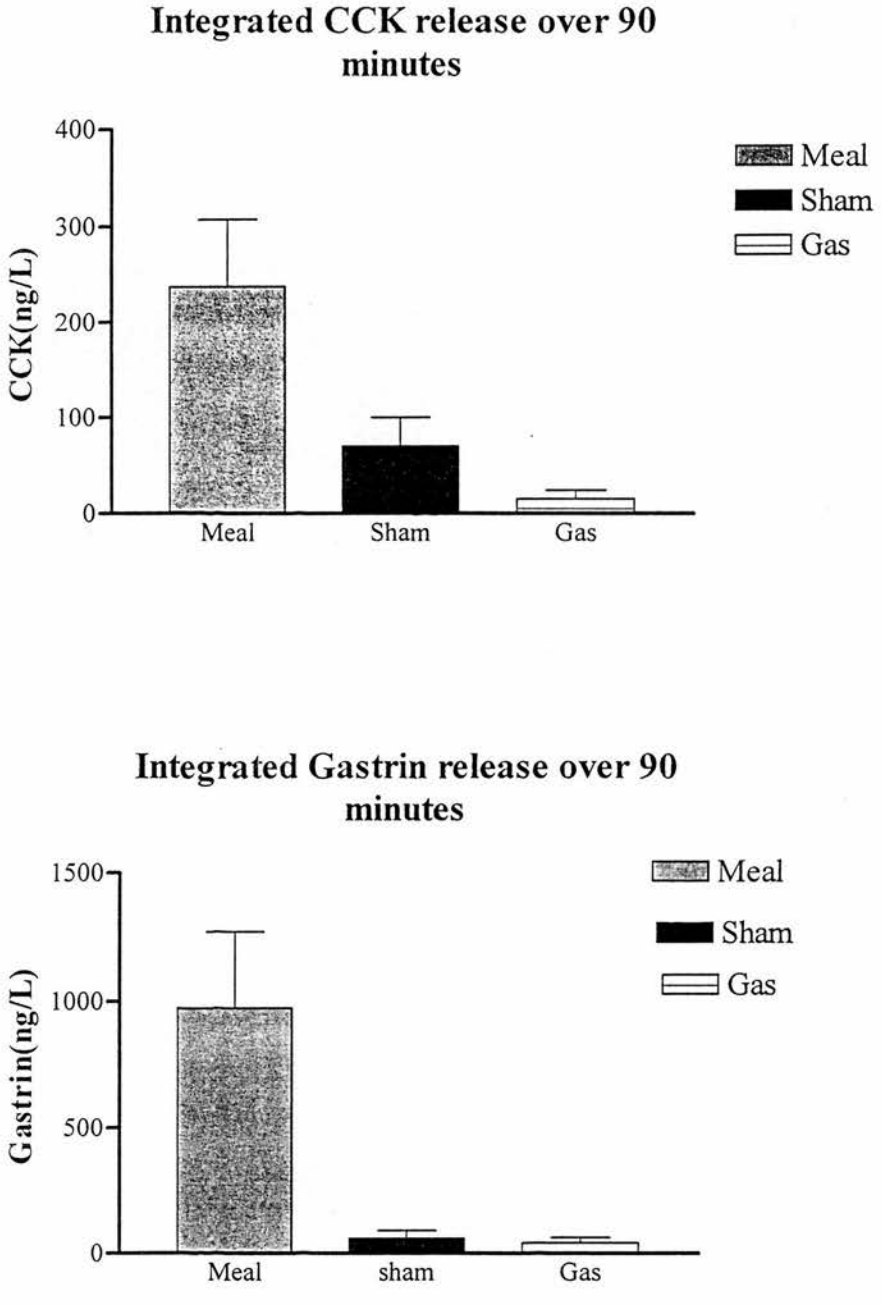


Figure 6.5. Integrated plasma CCK and gastrin concentrations (Means+SEM) during the different test procedures.

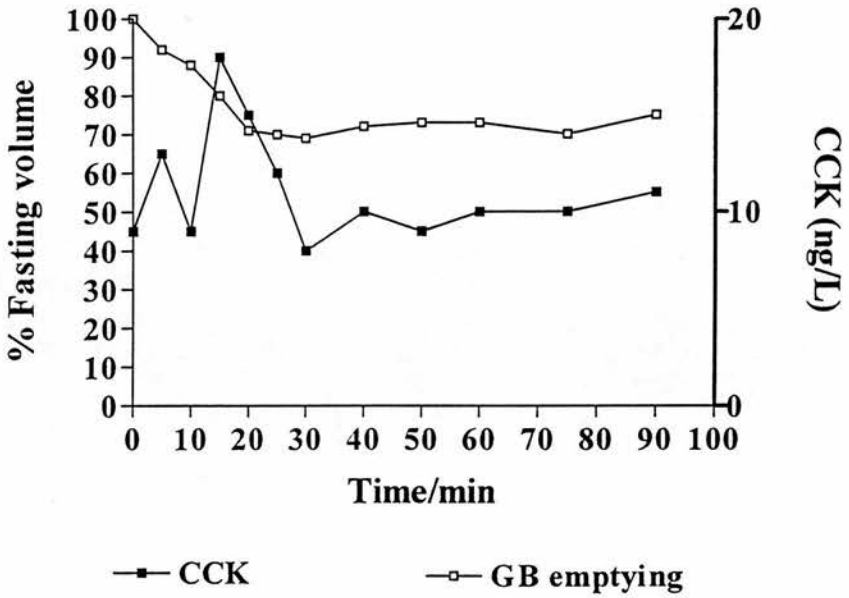


Figure 6.6 Average of rate of gallbladder emptying and plasma CCK concentration in two individuals who responded to sham meals.

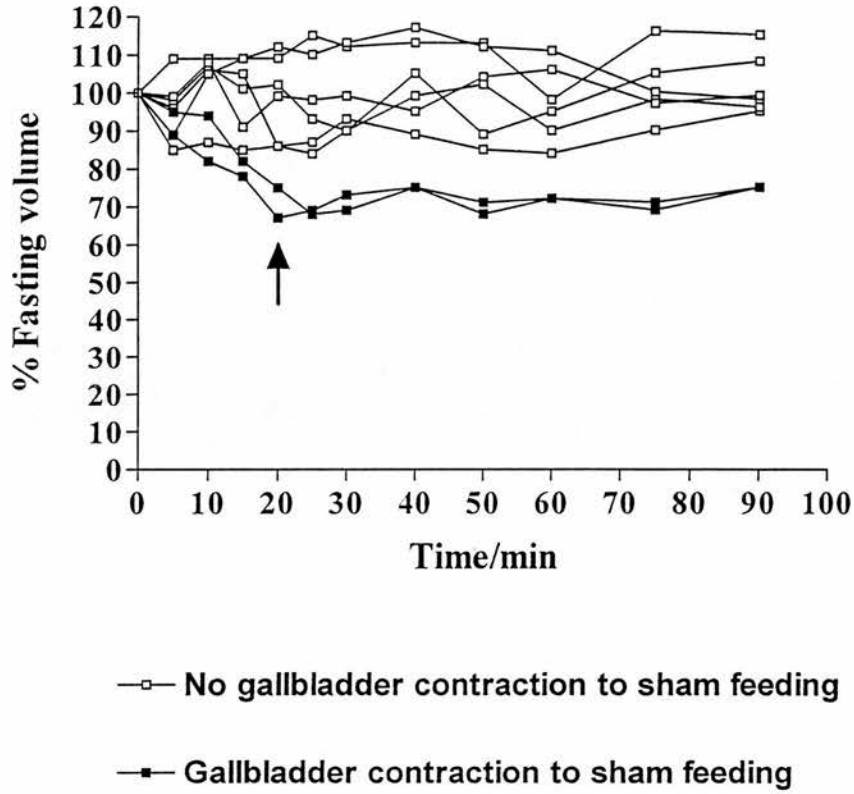


Figure 6.7 Gallbladder emptying in response to sham feeding for each volunteer.

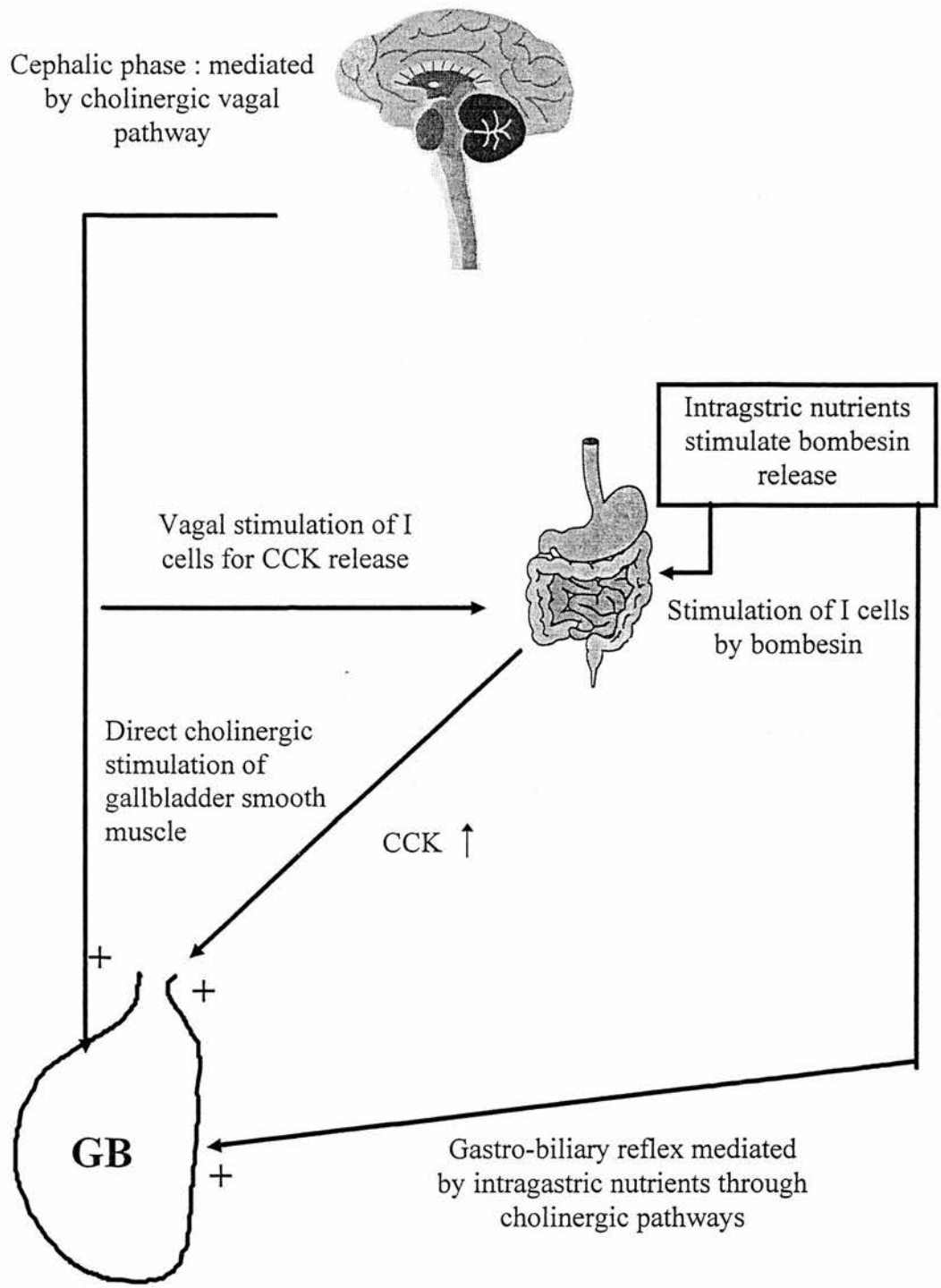


Figure 6.8. Diagrammatic representation of postulated mechanisms for early release of CCK and gallbladder emptying.

CHAPTER SEVEN

NITRIC OXIDE AND GALLBLADDER EMPTYING

7.1 Introduction

In this study the effects of NO donors and L-NMMA were defined in isolated gallbladder muscle strips, and gallbladders were stained for the presence of NOS with neuronal NOS specific antibody. Subsequently these agents were infused systemically during measurements of postprandial gallbladder emptying in a group of healthy volunteers.

7.2 Methods

7.2.1 *in vitro* study -

As described in section 5.4, bovine and human gallbladders were studied. Strips were incubated in Kreb's solution, and the effects of GTN, SNP, and L-NMMA upon muscle contraction were defined.

Ten fresh bovine gallbladders were obtained from a local abattoir and transported in cooled Kreb's solution. They were placed in a Petri dish under cooled oxygenated Kreb's solution and four muscle strips (10 mm long and 2-3 mm wide) were obtained from each gallbladder. Experiments were usually started within one hour but tissues were still found to be viable when used up to four hours after removal. Strips of gall bladder were mounted for isometric tension recording in four separate 10ml organ baths containing Kreb's solution at 37°C, continuously bubbled with O_2:CO_2 (95%:5%). The basal tension was adjusted to 2 g which was the tension that achieved maximum degree of contraction in response to 50 mmol potassium chloride in preliminary study. Changes in tension were monitored by isometric force transducers connected to a MacIntosh computer using MacLab Chart V3.2 software. The strips of muscle were equilibrated for at least one hour before the start of each experiment. The muscle strips were stimulated with CCK (Cholecystokinin, Ferring Pharmaceuticals, Malmo, Sweden) at concentration $5x10^{-9}$M to test for viability and to achieve contraction. In preliminary study with cumulative concentrations of CCK, we determined the EC_{50} at $5x10^{-9}$M and this concentration was used in further experiments. Once maximum tension was achieved, GTN (Glyceryltrinitrate, Nitrocine, Schwarz Pharma, UK. Final bath concentration= $5x10^{-5}$M), SNP (Sodium

nitroprusside, Sigma, St Louis, USA. 10^{-5} M) and 100 µl Kreb's solution as control was added into each bath. The effect of inhibition of NOS upon muscle contraction was examined by adding L-NMMA (Calbiochem-Novabiochem, U.K. Cumulative concentrations from 2.0 to 8.0 x 10^{4} M) to muscle strips in Kreb's solution under basal, unstimulated conditions.

The experiment was repeated using two normal human gall bladders removed at the time of pancreatic resection (Whipple's surgery). Immediately after removal, segments of gall bladder (2 cm long and 2 cm wide) were taken from the body and placed in a vacuum flask containing cooled pre-oxygenated Kreb's solution. Muscle strips were then studied in an identical manner to that described for the bovine gallbladder.

7.2.2 Immunohistochemistry of nitric oxide synthase

Ten archival gallbladders removed at laparoscopic cholecystectomy were subjected to immunohistochemical staining for NOS as described in section 5.7 In addition, two gallbladders were prepared by fresh frozen section immediately after cholecystectomy. Two colonic specimens and a normal appendix were prepared as positive controls. The colonic specimens were obtained from normal colon at least 5 cms from surgically removed carcinomata. Specimens were also stained with neuronal marker, PGP 9.5.

7.2.3 *in vivo* study -

(1) Subjects

Six young, healthy male volunteers (aged 23-31 years) were recruited after informed consent. All had a body mass index of less than 30 and none had diabetes, hyperthyroidism, liver disease or gallstones.

(2) Method of infusion

SNP (Faulding Pharmaceuticals plc, U.K.) and GTN (Nitrocine, Schwarz Pharma, UK) were infused as NO donors. Hydralazine (Ciba-Geigy, U.K.) was infused as a hypotensive control agent to ensure that any differences in gallbladder motility following SNP and GTN were not due to the nonspecific effects of vasodilation and hypotension. Finally, gallbladder emptying was studied during an intravenous infusion of the NOS inhibitor, L-NMMA (Calbiochem-Novabiochem, U.K.).

Each volunteer was studied after an overnight fast on five separate days in random order:

1) After a test meal consisting of a two egg omelette containing 10.8 g of fat and 147 kcal, consumed within 3 minutes. Intravenous normal saline (50 ml hourly) was infused as placebo.

2) After test meal and intravenous infusion of GTN increasing by increments of 1, 2, 5 and 10 mg/hour.

3) After test meal and intravenous infusion of SNP 0.5 micrograms/kg/min initially, then increased by 0.5 microgram/kg/min.

4) After test meal and intravenous infusion of hydralazine, 100 microgram/min, increasing by 50 microgram/min.

5) After test meal and intravenous infusion of L-NMMA, 3 mg/kg over 20 min. Previous study had shown that this dose of L-NMMA was well tolerated and increased mean arterial pressure by 10% [164].

After insertion of an indwelling intravenous catheter for blood sampling, volunteers were rested for at least 30 minutes until a stable resting blood pressure and heart rate were obtained. Infusion rates of the three hypotensive agents were adjusted at 15 minutes intervals until a decrease of mean arterial blood pressure of at least 10% or an increase of heart rate by 20% was achieved. Infusions of these agents or placebo were started at least an hour before the test meal and maintained until the end of the experiments. L-NMMA infusion was started 20 minute before the meal. The ultrasonographer was blinded to the infusates which were covered in aluminium foil. Blood pressure was measured at 15 minute intervals and heart rate was continuously

monitored by finger plethysmography using Finapres BP monitor machines (Ohmeda, Japan).

(3) Preparation of drugs

L-NMMA was purchased in ampoules of 200 mg which was constituted with normal saline to 50 ml. The reconstituted solutions were used within one hour after preparation. GTN was used unchanged at manufacturer's preparation of 1mg/ml in ampoule of 10mg. SNP was purchased in ampoule of 50 mg and this was diluted to 50 ml with 5% dextrose. The constituted solution was immediately covered with aluminium foil and used within three hours. Each 50 mg ampoule of hydralazine was dissolved to 50 ml with normal saline and used within three hours.

(4) Measurements of gallbladder volume

Measurements were taken before infusion (fasting volume) and just before ingestion of the fatty meal (pre-ingestion volume). The test meal was then ingested and for the next 30 minutes, gallbladder volumes were measured at 5 minute intervals. Subsequent measurements were taken at 10 minute intervals for a further 30 minute and at 15 minute intervals for the last 30 minutes. Blood pressures and pulse rates were monitored throughout the study at the same frequency as measurements of gallbladder volumes.

(5) Measurements of gut hormones

Blood samples were obtained from an intravenous catheter at the same time intervals as ultrasound scan measurements. The samples were kept on ice and plasma separated within 30 minute and stored at -70^{0}C for subsequent assay of CCK and gastrin. Gut hormones were measured by standard radioimmunoassay as described in 4.5.

(6) Statistical analysis

Measurements of gallbladder volumes and gut hormones were performed in duplicate, and the mean of these two measurements was used for further analysis of results. Gallbladder volume was expressed in millilitre and as a percentage of fasting

volume. Ejection fraction (%) was calculated by [(fasting volume-volume at time 't')/fasting volume x 100].

Integrated CCK and gastrin concentrations for each individual volunteer were determined by calculating the area under the CCK and gastrin concentration time curves after subtraction of basal values. Results for the group are expressed as mean±SEM. Statistical analysis was performed by analysis of variance. Differences with a two-tailed $p<0.05$ were considered significant.

For the *in vitro* experiment, mean tension was recorded in gram and results expressed as mean±SEM. Changes in tension with different reagents were compared by paired 't' test.

The studies of gallbladder contraction were approved by the Lothian Ethical Subcommittee for Medicine and Oncology.

7.3 Results

7.3.1 *in vitro* study of gallbladder contraction

Some strips from bovine gallbladders exhibited spontaneous tonic and phasic muscular activity in the absence of CCK; in others contraction only occurred after the addition of CCK. Mean basal resting tension was 2.5±0.4 g (6 specimens) and this increased to 4.2±0.6 g after CCK. Subsequent addition of 100 µl of Kreb's solution did not alter tension. In contrast GTN and SNP immediately reduced mean tonic activity to 1.3±0.1 g ($p<0.01$) and 1.4±0.2 g ($p<0.01$) respectively; addition of both drugs also abolished phasic contractions for approximately 1 minute (figure 7.1). Figure 7.2 shows a typical polygraph showing contractile activity of muscle strips and abolition of tone and phasic activity after addition of GTN and SNP. L-NMMA increased mean tonic contraction from 2.7±0.2 g (4 specimens) to 3.6±0.3 g ($p<0.05$). The maximum effect was associated with concentration of L-NMMA of $5x10^{-4}$ M.

In the human gallbladders, CCK increased mean basal tension from 1.2 g to 1.7 g. Tonic and phasic activity were inhibited by GTN and SNP. Both agents decreased mean basal tension to under 1.0 g. L-NMMA increased mean tension from 1.1 to 1.5. g.

7.3.2 Immunohistochemistry

Positive staining for PGP at dilution of 1/600, was obtained on the myenteric and submucosal plexus of colon and appendix. Staining was obtained in both the neuronal cell bodies and fibres, and in fibres within the smooth muscle. In comparison with colon and appendix, gallbladders were more sparsely populated with nerves although ganglion cells and nerve fibres could be easily identified in all layers of the gallbladder wall with PGP (see plate 7.1). Large trunks in the gallbladder subserosa and slender filaments in the mucosa were both strongly positive for PGP. Neurons were also seen in the lamina propria.

NOS staining was identified in the myenteric and submucosal plexus of both colonic and appendix sections with antisera to NOS at 1/400 concentration (see plate 7.2). Neuronal NOS immunoreactivity was observed consistently in all the ganglion cells and nerve fibres of plexus myentericus and most of the nerve fibres of the circular muscle layer. Immunoreactive neurons were much less conspicuous in the longitudinal muscular layer. This concentration of NOS antiserum was therefore used for subsequent staining of gallbladders. No NOS immunoreactive neurons could be identified in the gallbladder although as in the colon, background immunoreactivity was visible in erythrocytes and platelets (see plate 7.3). Similar negative staining was observed in both archival and fresh frozen specimens.

7.3.3 *in vivo* study

The median dose of drugs infused is shown in table 7.1. During infusions with the three hypotensive agents, there was a significant decrease in mean blood pressure and an increase of heart rate. All of the subjects also experienced headache and facial

flushing but none developed nausea or vomiting. There was no significant change in the fasting gallbladder volume after one hour of infusion (table 7.2).

The rates of gallbladder emptying during infusions with placebo, hydralazine and L-NMMA are shown in figure 7.3. There were no significant differences in the rates of gallbladder emptying during infusion with these three agents. Figure 7.4 shows the rate of gallbladder emptying during infusions with GTN, SNP and placebo. During infusion with both GTN and SNP, there was significant impairment in gallbladder emptying. At the end of the study period, the ejection fraction was 50% compared with the ejection fraction in excess of 80% during infusion with placebo. There was no significant difference in the integrated CCK and gastrin concentrations during the post-prandial period with all the different infusions (figure 7.5). The concentration profiles of CCK and gastrin during the post-prandial period were very similar.

7.4 Discussion

In isolated bovine and human gallbladder muscle strips, CCK stimulated tonic and phasic activity was inhibited by the NO donors GTN and SNP. In contrast the NOS inhibitor L-NMMA increased muscle tone. These *in vitro* effects correspond to *in vivo* findings of impaired post-prandial gallbladder emptying during infusions with GTN and SNP. This inhibition was not due to hypotension as hydralazine did not cause similar inhibition. Post-prandial CCK and gastrin release were not influenced by infusions of the NO donors. It is therefore reasonable to conclude that the NO pathway may be involved in gallbladder motor function.

The *in vitro* observations described in these studies are consistent with those of Mourelle et al, who showed that NANC pathway may influence gallbladder function in guinea pigs. In this study, resting gallbladder pressure of anaesthetised guinea pigs and its CCK induced contractions were enhanced by inhibition of NOS with L-NAME; this effect of L-NAME could be reversed by L-arginine [262].

Whilst the *in vivo* study showed that GTN and SNP significantly impaired post-prandial gallbladder emptying, fasting gallbladder volumes were unaffected by these infusates. This is probably because the fasting gallbladder is maximally dilated. In contrast to the observation by Konturek et al [267], L-NMMA did not cause reductions in fasting gallbladder volumes or accelerated gallbladder emptying. The *in vitro* study with isolated muscle strips showed that under basal condition, L-NMMA had a small but significant excitatory effect on gallbladder contraction although the increase in tone contraction was much smaller than that induced by CCK. The reason for this discrepancy is unclear but it is possible that the increase in tone and contractility caused by L-NMMA during the *in vitro* experiment was due to stimulation of cNOS (endothelial) during preparation of the muscle strips for the experiment. Acetylcholine, bradykinin or shear stress can cause influx of Ca^{2+} into endothelial cells and results in NO synthesis [274]. Bacterial lipopolysaccharides in the Kreb's solution is known to induce the NOS in vascular tissues *in vitro* [179]. On the other hand, the lack of effect on gallbladder motility during *in vivo* experiment can be attributed to the dose and method of infusion of L-NMMA. L-NMMA was only infused to the volunteers before meals in contrast to the study by Konturek et al [267] in which it was continuously infused throughout the post prandial period.

Both SNP and GTN release NO by pathways which are independent of NOS and the L-arginine : NO pathway [374]. Unlike L-arginine, they do not stimulate release of glucagon and somatostatin which may have secondary effect on gallbladder emptying [262]. The impaired post-prandial gallbladder emptying by GTN and SNP was not associated with any effects upon the post-prandial release of gastrin and CCK. Therefore the inhibitory effects of these infusates upon gallbladder emptying were not due to changes in the release of these gastrointestinal hormones. Furthermore, the impairment of gallbladder emptying was not a secondary effects of hypotension as hydralazine infusion did not induce similar inhibition despite inducing similar degree of hypotension.

It is possible that the effects of the NO donors upon gallbladder emptying were indirect and mediated through delayed gastric emptying. Sublingual GTN and intravenous L-arginine have been shown to delay gastric emptying in a group of healthy volunteers after a liquid [216] or a semi solid meal [375]. In this study gastric emptying was not measured, but the observation that plasma gastrin and CCK concentrations following the meals were similar during each study day suggest that this was not an important factor.

The presence of NANC inhibitory nerves and the role of NO as a neurotransmitter for gallbladder motor function are unclear. For example, Li et al reported that transmural EFS had no effect upon gallbladder muscle contraction in the prairie dog [376]. Furthermore, Feeley et al failed to demonstrate NANC activity in isolated human gallbladders which were subjected to EFS following adrenergic and cholinergic blockade [265]. In contrast, McKirdy et al were able to detect NANC inhibitory responses in isolated human gallbladders using a shorter pulse width of EFS and noted this was abolished by L-nitroarginine [266]. L-arginine reversed the effects of L-nitroarginine, suggesting a role for NO in NANC neurotransmission in the human gall bladder.

To clarify this controversial area, attempts were made to stain the synthetic enzyme NOS in human gallbladder muscle. Control tissues (colon and appendix) stained positively for neuronal NOS antisera on nerve cell bodies and fibres. Background immunoreactivity with NOS within erythrocytes and platelets was also noted in both the control tissues and gallbladder sections. However, NOS could not be demonstrated on nerve cell bodies and fibres of archival and fresh human gallbladders. That nerve bodies and axons were present within these specimens, was confirmed by positive staining for PGP which is known to be a reliable marker for axons [365]. Similar observations of background immunoreactivity with vascular tissues have been reported by Springall et al [287] with antibodies to rat brain NOS. The antibody was similar to the one used in the current study in that they were both raised in rabbits to the carboxyl-terminal peptide 58, corresponding to amino acids

1409-1429 of rat brain enzyme. It is likely that NO activity in homogenates of guinea pig gallbladders [262] were due to activity within vascular rather than neuronal tissues.

The role of NO may be important in chronic cholecystitis and could account for the impaired motility in this disease. Increased levels of inducible NOS were found in inflamed gallbladders and immunohistochemistry showed localisation of NOS in macrophages [126]. It is theoretically possible that during chronic cholecystitis, the generation of NO by the inflammatory process locally could inhibit gallbladder smooth muscle contraction.

In conclusion, pharmacological doses of exogenous NO donors impaired postprandial gallbladder contraction *in vivo* and relaxed contraction of isolated muscle strips. However, it is unlikely that NO is the neurotransmitter for NANC innervation in the gallbladder as its synthetic enzyme could not be identified in either gallbladder nerve bodies or fibres.

Table 7.1 Change in mean blood pressure and heart rate with the different infusions.

Drug	**Median Dose**	**Mean blood pressure±SEM (mmHg)**		**Mean heart rate±SEM (per minute)**	
		Pre-infusion	Post-infusion	Pre-infusion	Post-infusion
GTN	60 μg/minute	76±2	67±2*	60±4	71±5*
SNP	0.5μg/kg/min	76±2	65±3*	55±3	70±6*
Hydralazine	100 μg/min	70±3	61±1*	57±3	61±4*
L-NMMA	3 mg/kg	70±2	81±3*	60±3	51±3*
Placebo	3 ml/min	75±2	75±2	55±2	57±3

* $p<0.05$ (paired student t test)

Table 7.2. Mean fasting and pre-ingestion gallbladder volumes (ml)+SEM.

	Fasting	**Pre-ingestion**	**p-value***
GTN	18±3	20±3	NS
SNP	21±4	21±4	NS
Hydralazine	22±3	20±3	NS
L-NMMA	21±3	18±3	NS
Placebo	19±2	20±2	NS

* paired student t test

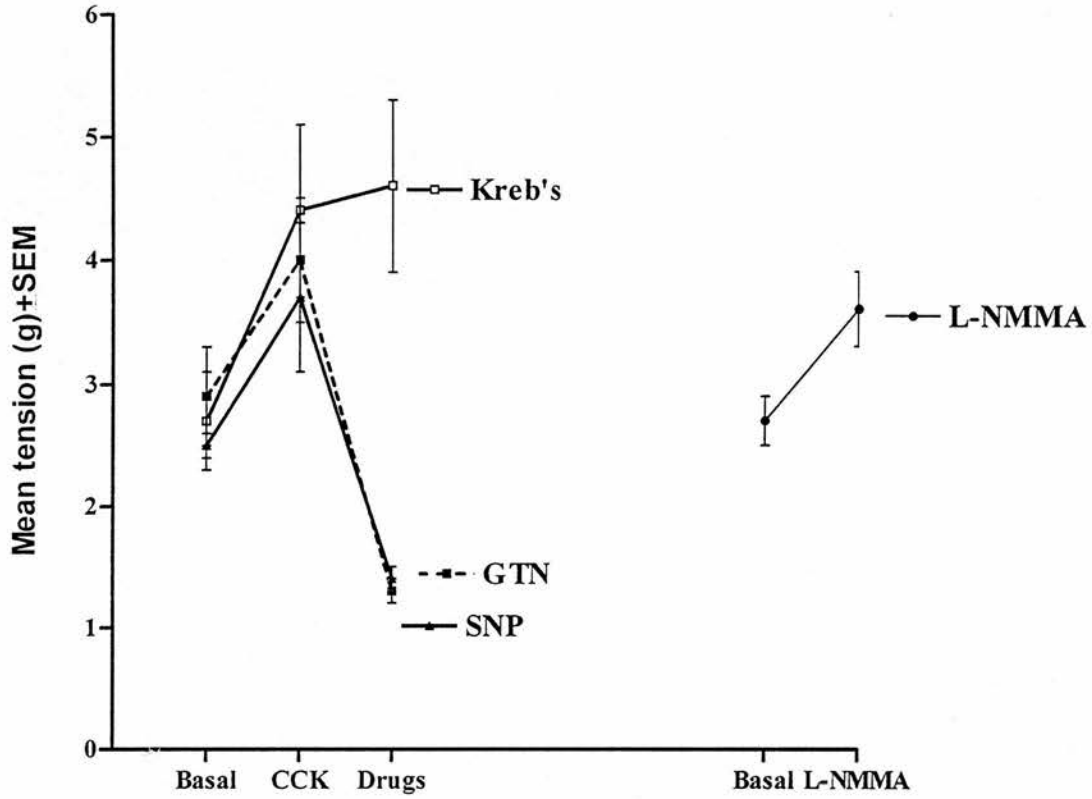


Figure 7.1. Change of tension of bovine muscle strips.

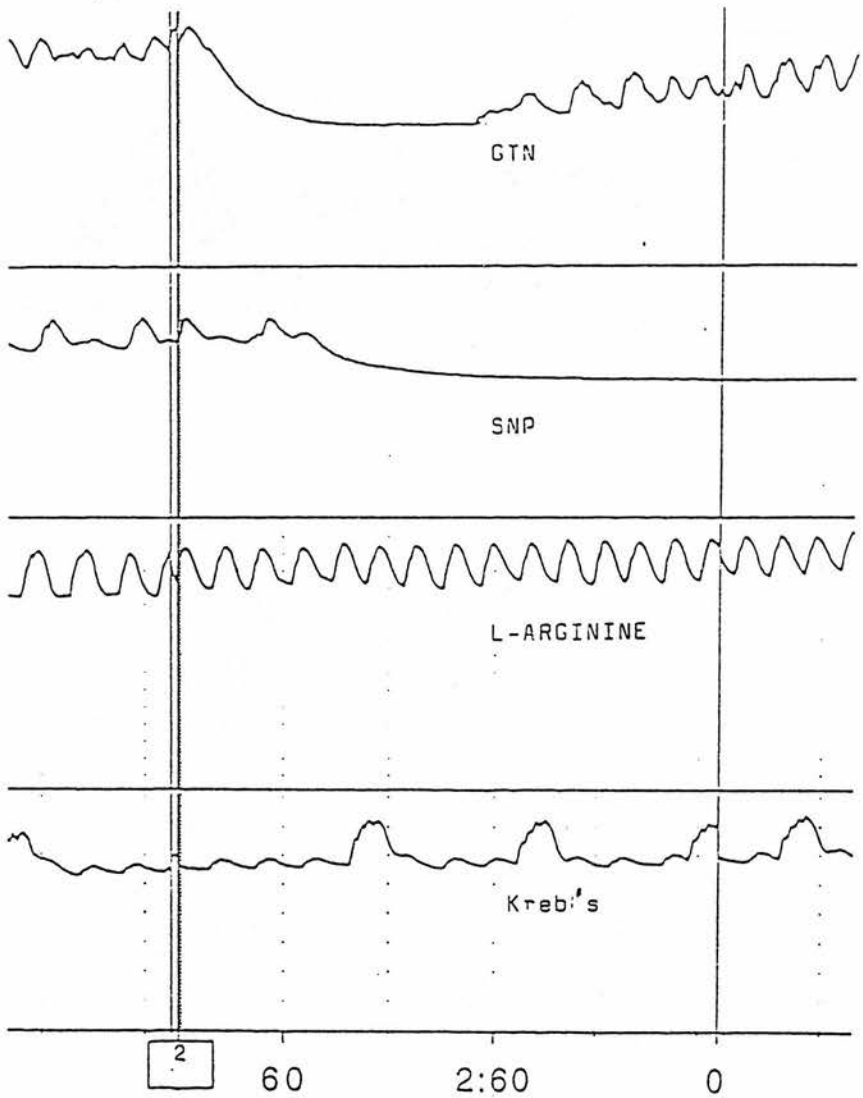


Figure 7.2 Polygraph shows muscular contractile activity and abolition of tone and phasic activity after GTN and SNP.

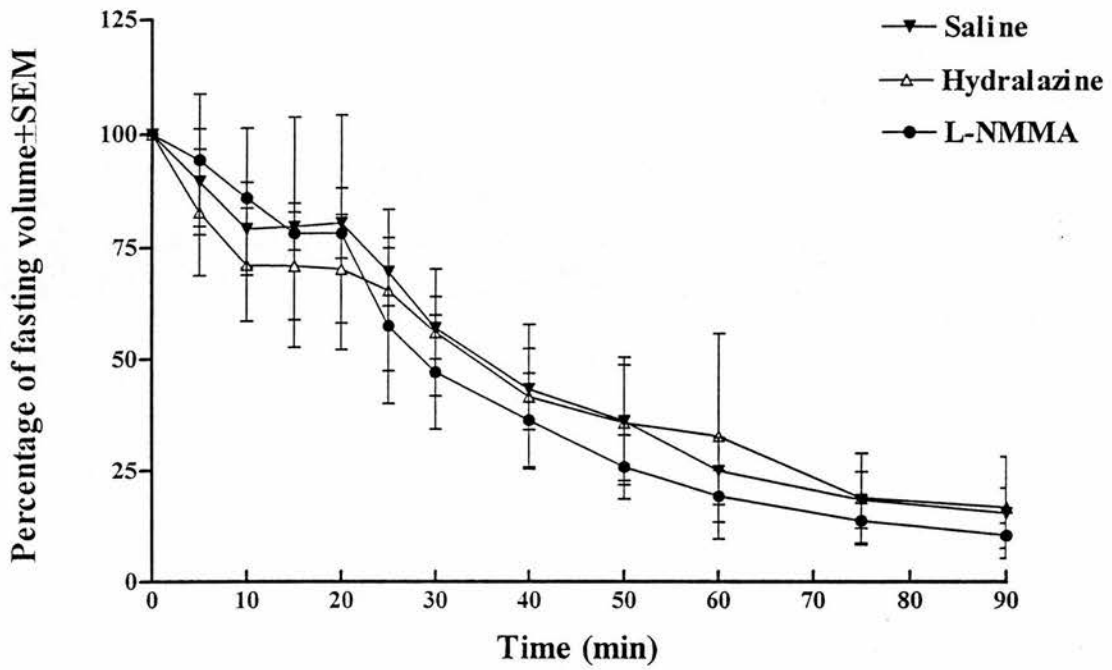


Figure 7.3 Gallbladder volume expressed as percentage of fasting volume over time after ingestion.

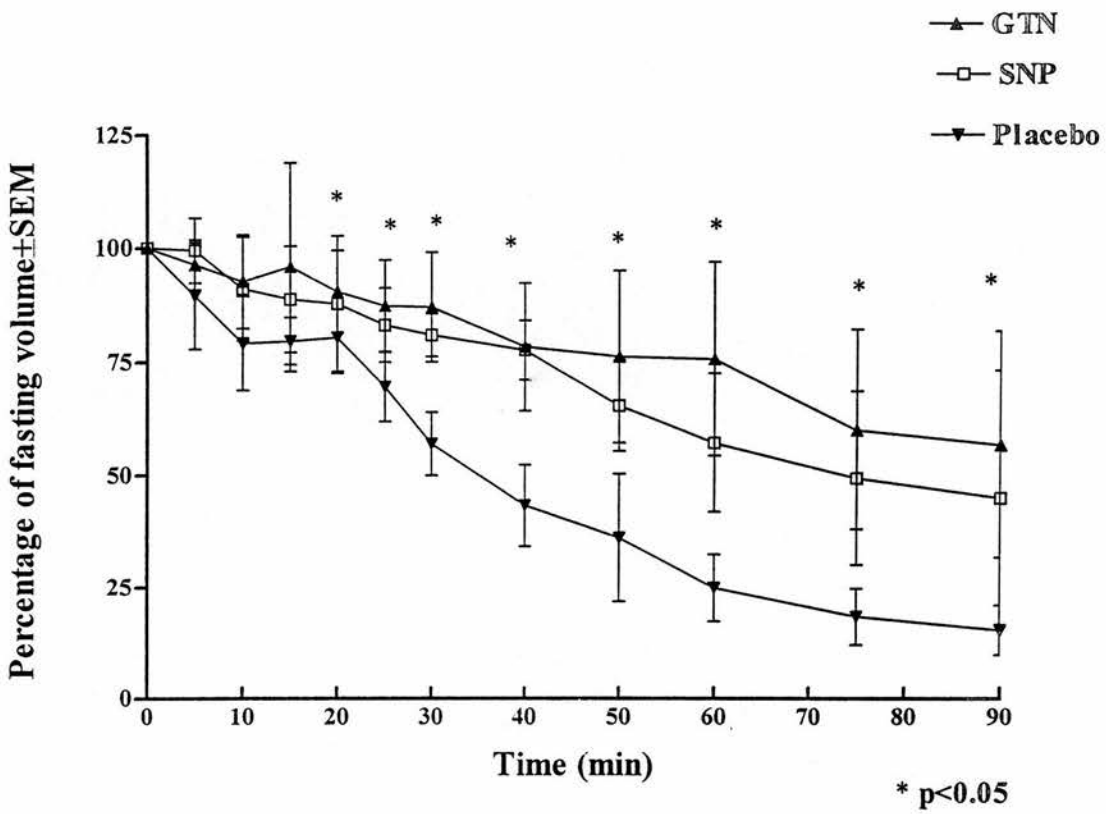


Figure 7.4 Gallbladder emptying during infusions with GTN, Sodium nitroprusside and placebo.

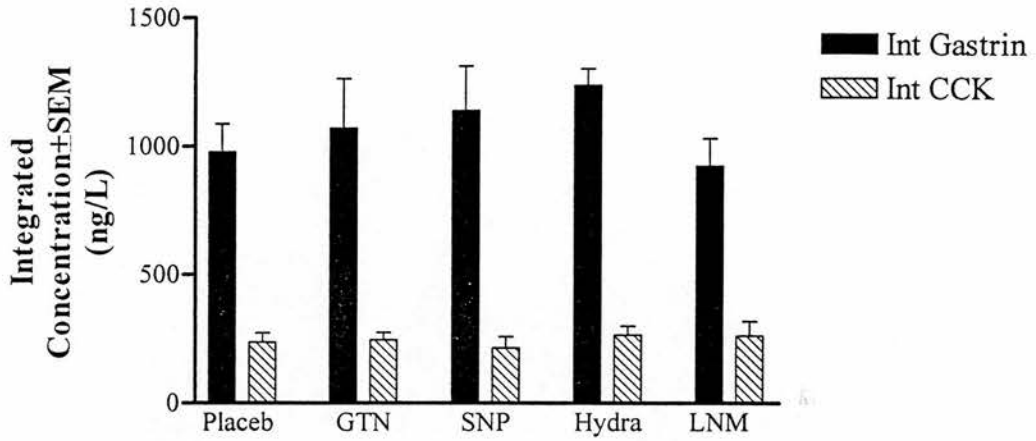


Figure 7.5 Integrated CCK and gastrin concentrations over 90 minutes.

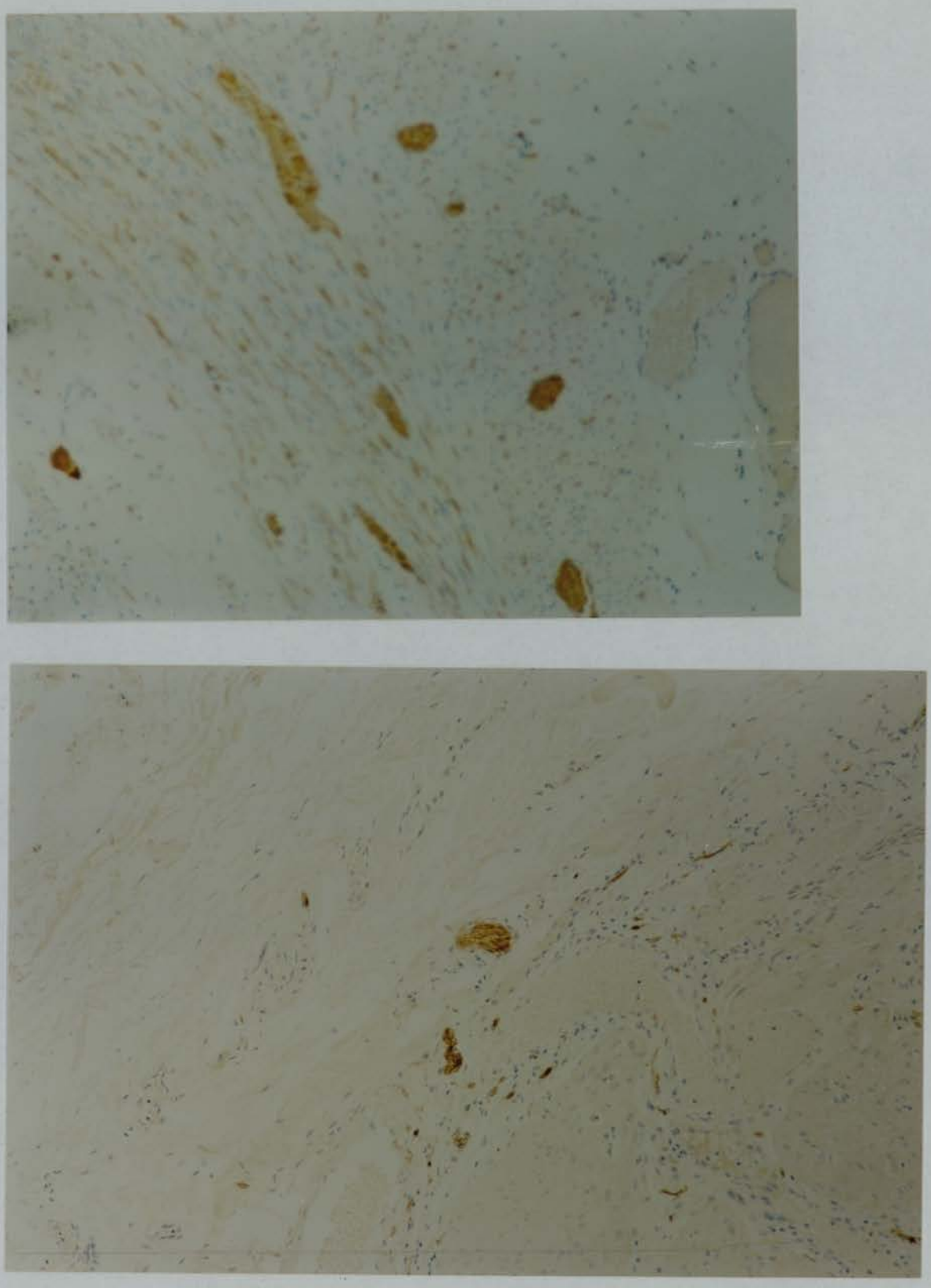


Plate 7.1 Staining of ganglion cells and nerve fibres with PGP in gallbladder wall.

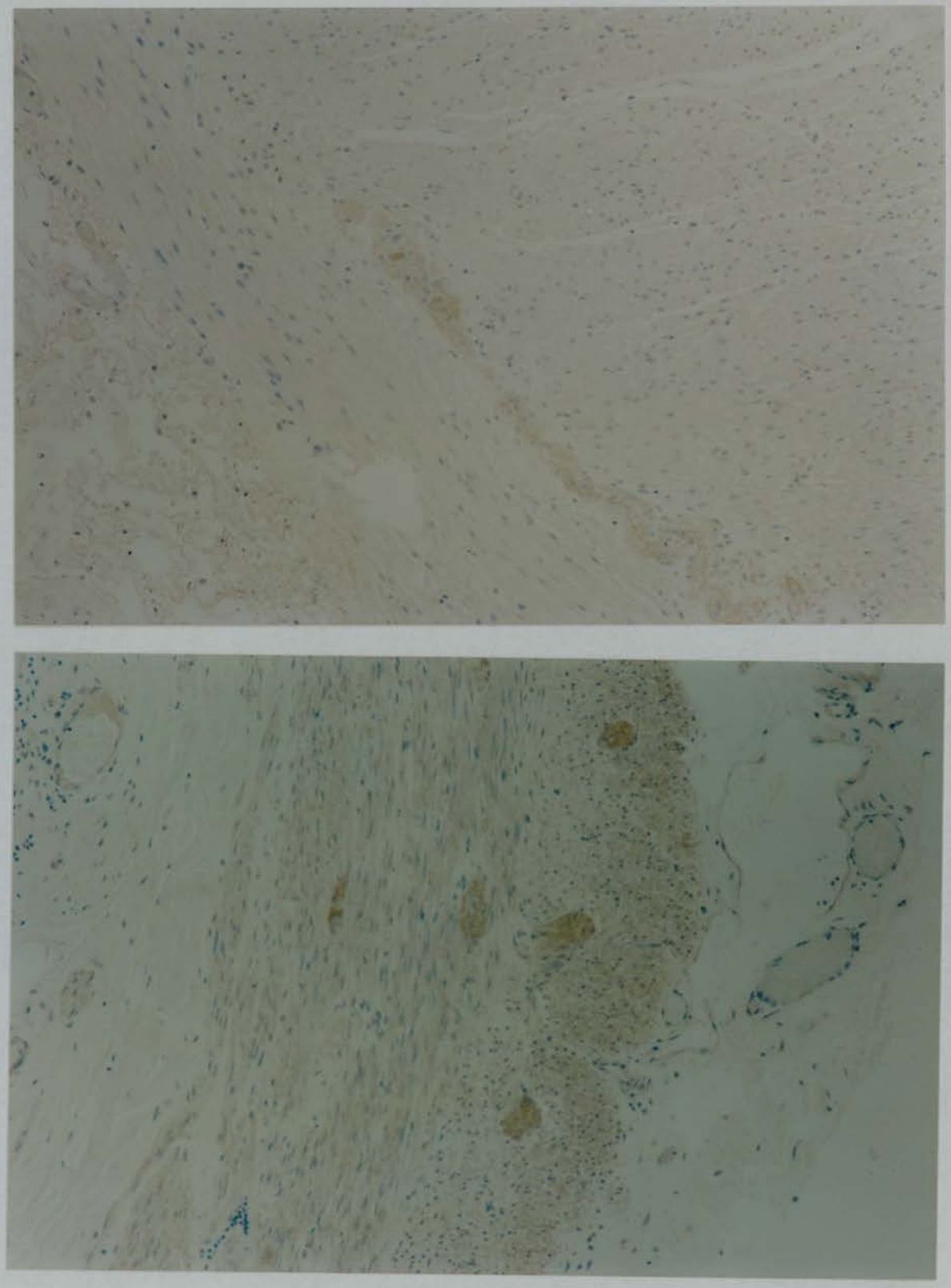


Plate 7.2 NOS staining in the myenteric and submucosal plexus of colonic (top plate) and appendix (bottom plate)

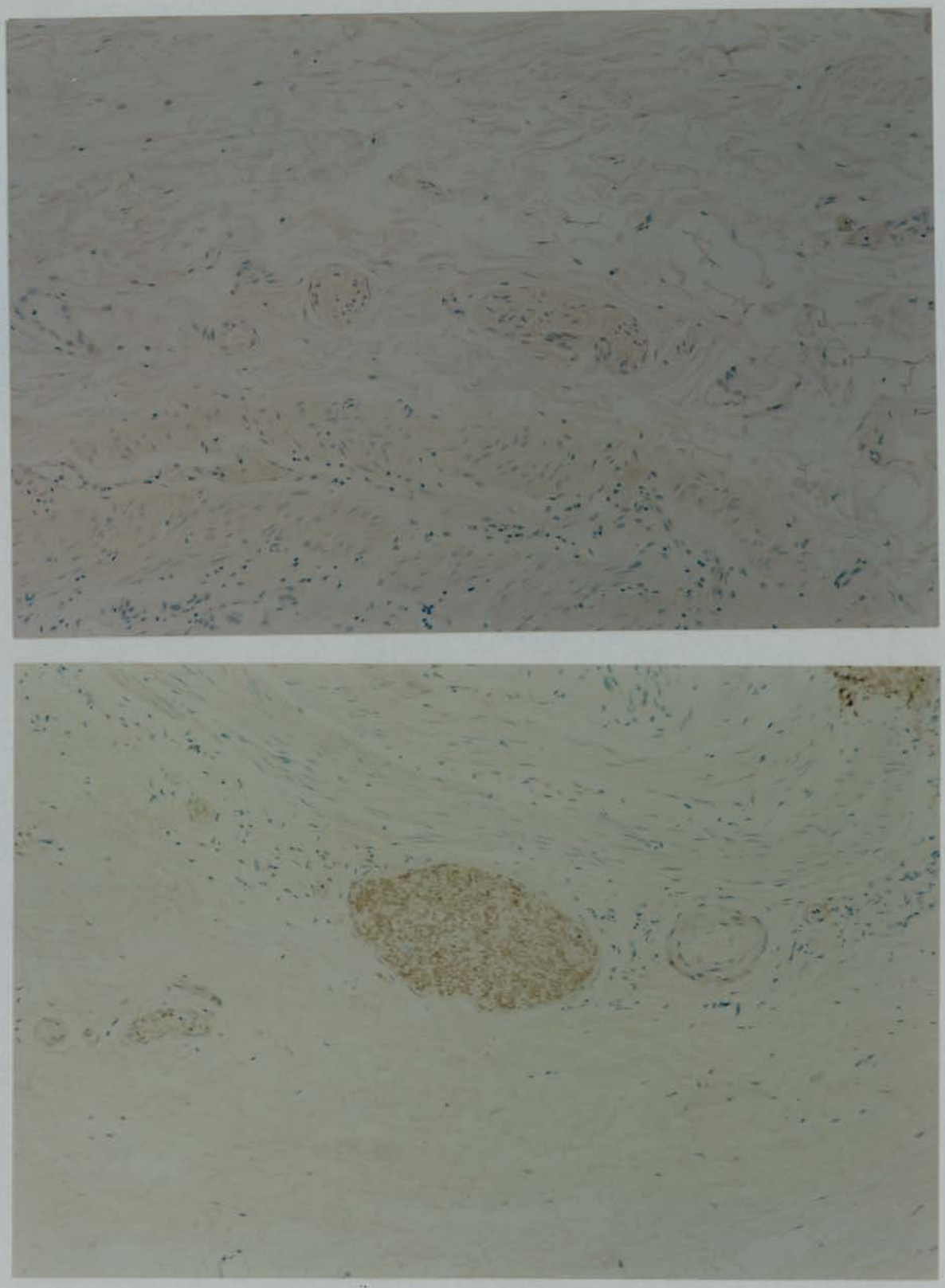


Plate 7.3 No NOS staining was obtained in the gallbladder neurones (top) though background immunoreactivity was visible in erythrocytes and platelets (bottom).

CHAPTER EIGHT

NITRIC OXIDE AND THE SPHINCTER OF ODDI

8.1 Introduction

Delivery of bile into duodenum is controlled by hepatic bile secretion, gallbladder contraction and the SO. CCK is believed to be responsible for post-prandial gallbladder contraction and SO relaxation. Recent studies have shown that the effect of CCK upon the SO is mediated through stimulation of the nonadrenergic noncholinergic (NANC) nerves [10] and nitric oxide (NO) is an important element of this pathway [201-203].

There have been few studies on the effect of NO on the function of the SO. Slivka et al localised the presence of NO synthase (NOS) in nerve fibres and bundles of human SO and demonstrated that topical application of *S*-nitroso-*N*-acetylcysteine (SNAC), a NO donor, inhibits SO motility. However, SNAC needs to be freshly prepared, is not stable over a prolonged period of time and is not widely available. The aim of the present study was to examine the effect of glyceryl trinitrate (GTN, Nitrocine, Schwarz Pharma, U.K.), a form of NO donor that is commercially available, on SO motility.

8.2 Subjects and Method.

8.2.1 Patients characteristics

Nineteen patients (17 females; median age 41, 29-61 years) undergoing routine SO manometry for investigation of upper abdominal pain were examined. Seventeen patients underwent manometry for investigation of post-cholecystectomy pain occurring one to five years after surgery. The other two patients had intact gallbladders. None of the patients was on regular medication with nitrates or calcium channel blocker drugs. All of the patients had normal abdominal ultrasound scans and ERCP prior to manometry.

8.2.2 Sphincter of Oddi manometry

After an overnight fast, patients were sedated with 5-10 mg of intravenous midazolam. SO manometry was performed with a standard triple-lumen

polyethylene manometric catheter by the water perfusion technique as described in section 5.2.2.

After recording duodenal pressure, which was taken as the zero reference, the papilla was cannulated and the catheter was withdrawn across the SO in 2 mm increments using the black marks on the catheter as a guide. Recordings were obtained for at least 60 second at each station. After two such pull-throughs, the catheter was repositioned so that the proximal and middle channels were recording phasic SO contractions. The most distal channel was therefore located just outside the papilla. Patients were randomised into three groups. The first group of patients received topical application 10ml normal saline; the other two groups received GTN at either 5 mg (0.5 mg/ml with volume of 10 ml; GTN 5) or 10 mg(1mg/ml with volume of 10 ml; GTN 10). The distal channel was disconnected from the perfusion system and the drug solutions were then infused into the channel over 1 minute. Recording was started immediately at the end of drug infusion and continued for 5 minutes.

8.2.3 Analysis of manometric recordings

Manometric tracings were coded and analysed by an experienced observer who was blinded to the types of infusion.

8.2.4 Statistical analysis

All data are reported as mean±SEM unless otherwise stated. Paired Student's tests were used to compare variables before and after injection. Differences with P values of less than 0.05 were considered significant.

The studies were approved by the Lothian Ethical Subcommittee for Medicine and Oncology.

8.3 Results

Five patients were randomised to saline injection; two groups of seven patients were each randomised to either 5 mg or 10 mg GTN.

The baseline results of SO motor activity are presented in table 8.1. The means of all the motor variables were within normal limits as defined by previous studies using a similar perfusion technique in healthy volunteers [346,350,353]. Two patients were found to have SO dysfunction [377]. One patient exhibited a basal SO tonic pressure of 90 mmHg accompanied by tachyoddia (frequency of 12 contraction per seconds); she was randomised to GTN 5 mg. The second patient who was randomised to 10 mg GTN was also found to have tachyoddia, but had normal tonic SO pressure.

There was a non-significant increase in basal SO tonic pressure, amplitude and frequency of phasic activity after injection of 10ml normal saline. In contrast, mean tonic SO basal pressure and all the variables of phasic SO activity significantly diminished after injection with 5mg and 10mg of GTN; specifically phasic contractions became significantly less frequent, were shorter in duration and had decreased amplitude (figure 8.4 for SO manometry tracing). Consequently the motility indices fell from 312±35 mmHg sec to 39.5±19 in the group who received GTN 5 mg and from 462±125 to 57±27 mmHgsec in the higher dose GTN group (figure 8.1, 8.2 and 8.3). These effects were seen both in patients who had normal SO function and in those who had abnormal SO motility.

The duration of GTN inhibition on SO was 2 minutes. Although duodenal motility was not quantitated, video recording also showed cessation of contractility.

8.4 Discussion

Topical application of GTN but not normal saline significantly inhibited SO tonic and phasic activity. Five and 10 mg of GTN showed similar efficacy with a duration of approximately 2 minutes in both cases.

Complex neurogenic and hormonal mechanisms are involved in the control of the SO muscle tone and motility. Ingestion of a meal [60] and injection of CCK [61] produced a decrease in SO basal pressure and amplitude and reduced the frequency of phasic

contractions, thereby facilitating bile flow into the duodenum. The inhibitory effect of CCK is believed to be mediated through NANC inhibitory nerves [10]. NO has been demonstrated to control SO motility in animals [269,270,273,349,378] and the presence of NOS has been confirmed in the SO neurons of rabbits [273,378,379]. In humans, involvement of NO in SO motility is suggested by the findings of NOS in nerve bundles and fibres by NADPH diaphorase immunohistochemical staining and, furthermore topical application of SNAC inhibits SO motility [273].

In humans, sublingual GTN has been found to be effective in improving post-cholecystectomy pain associated with SO dysfunction although side effects limited its therapeutic potential [380]. Staritz et al demonstrated that sublingual GTN lowered SO basal pressure and contraction amplitude during SO manometry. In contrast to findings from this current study, SO frequency was not inhibited but this difference could be attributed to drug dosage and mode of administration [271]. Staritz et al subsequently showed that common bile duct stones between 6-12 mm in diameter could be removed from an intact papilla after GTN-induced SO relaxation [272]. Recently, sublingual GTN spray was shown to facilitate papillary cannulation during ERCP [381].

Topical application of GTN could be of clinical application during ERCP as it inhibits both duodenal and SO motility. When administered orally, sublingual GTN can cause headache and may cause hypotension. Local administration to the papilla was not associated with systemic effects and high volumes can be targeted to the SO. This may have advantages over the administration of systemic drugs. It would be helpful in inhibiting duodenal and SO motility in patients who are intolerant of buscopan or glucagon during ERCP. It could also facilitate extraction of small stones from the biliary tree without resort to sphincterotomy or papillary balloon dilatation. However, its duration of action is relatively short and this may limit its clinical application.

In summary, topical application of GTN inhibited SO tonic and phasic contraction. This mode of GTN delivery may be of clinical application during ERCP cannulation and stone extraction and should be examined in clinical trials.

Table 8.1 Baseline manometric motor variables (Means+SEM) before application of drugs.

	Saline (n=6)	**GTN 5 (n=7)**	**GTN 10 (n=7)**
Mean Duodenal pressure (mmHg)	25.7±4.4	22.7±3	22.5±3.4
Mean CBD pressure (mmHg)	15.1±3.8	6.2±4.3	10.4±2.7
SO motor characteristics			
Basal (mmHg)	31.1±1.8	16.7±2.7	33.2±4.3
Amplitude (mmHg)	108.8±26	77.8±7	84±18
Duration (seconds)	5.6±1.4	4.8±0.3	5.4±1.6
Frequency (per minutes)	5.7±1.8	4.4±0.3	7.2±1.5
Motility Index (mmHg sec)	496±118	312±35	462±125

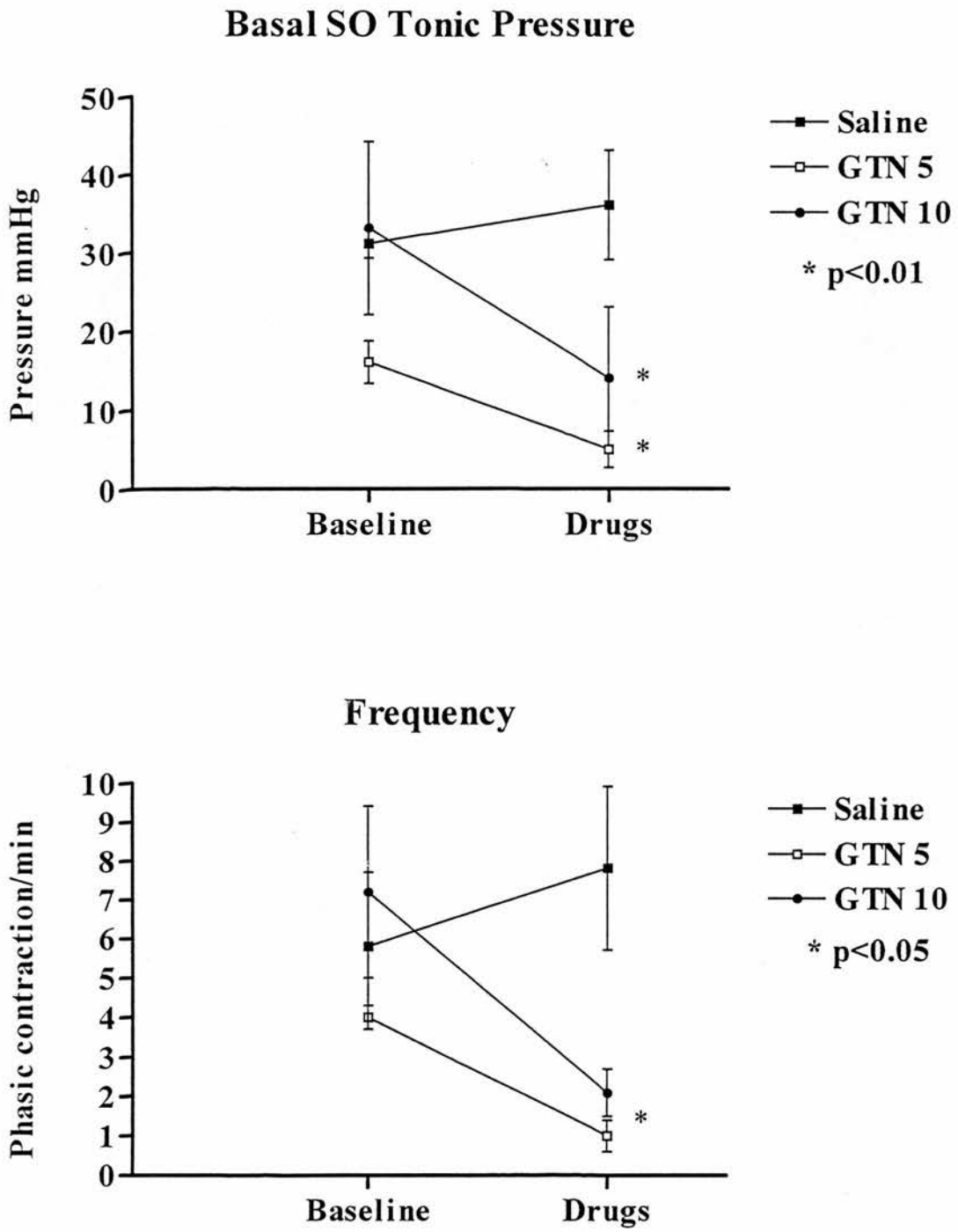


Figure 8.1 Change in basal SO tonic contraction and and frequency of phasic contractions (Means + SEM) with the different infusions.

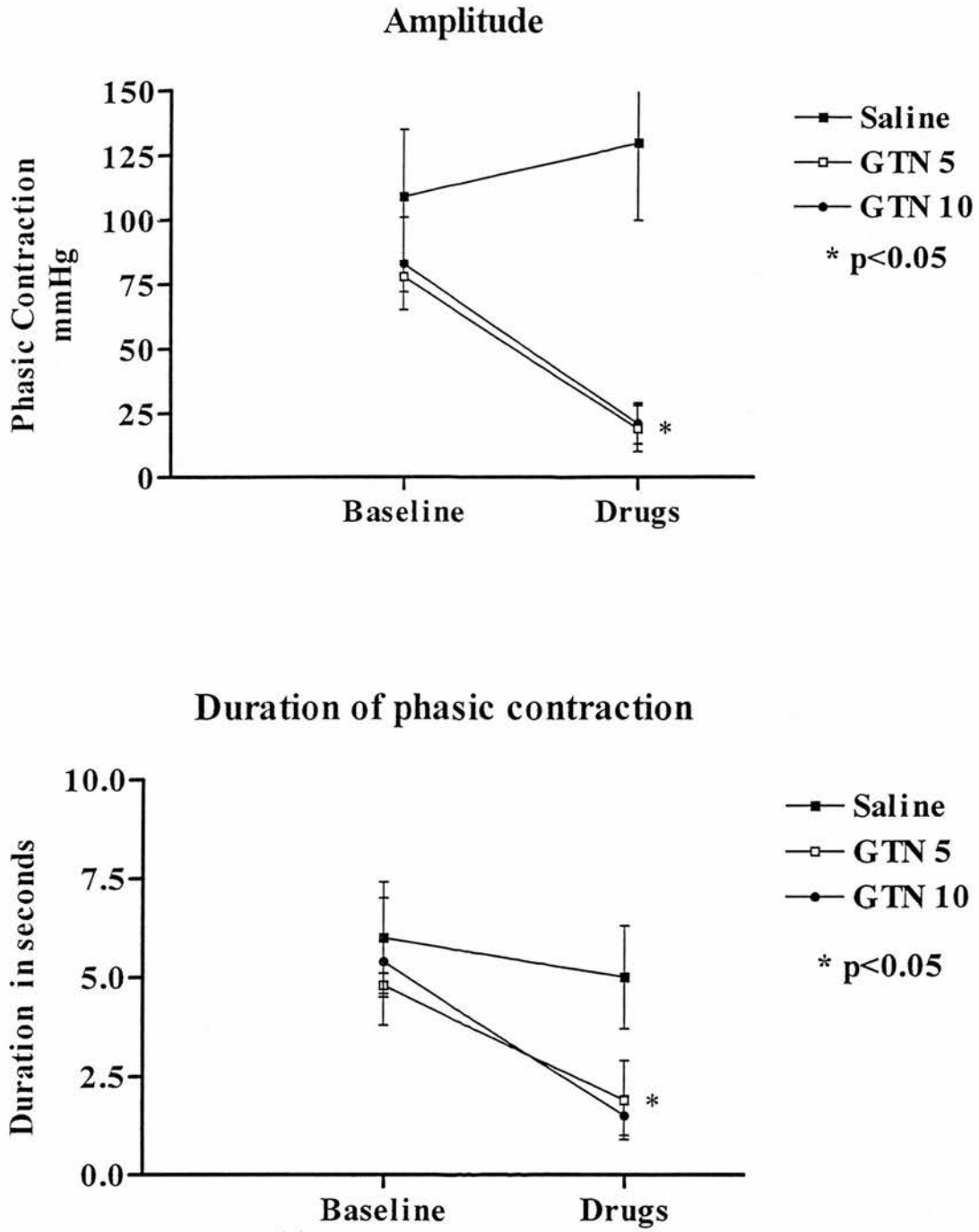


Figure 8.2 Change in amplitude and duration of phasic contractions (Means+SEM) with the different infusions.

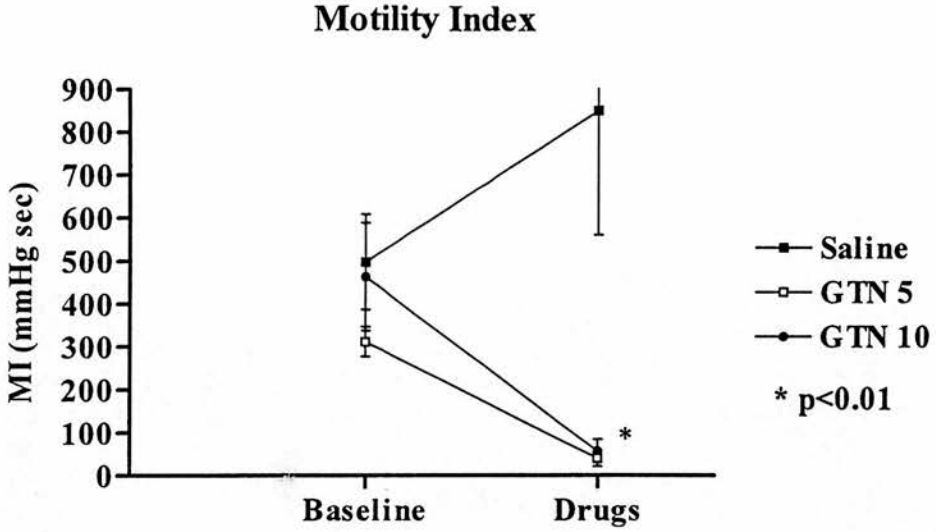


Figure 8.3 Change in motility index (Mean+SEM) with the different infusions.

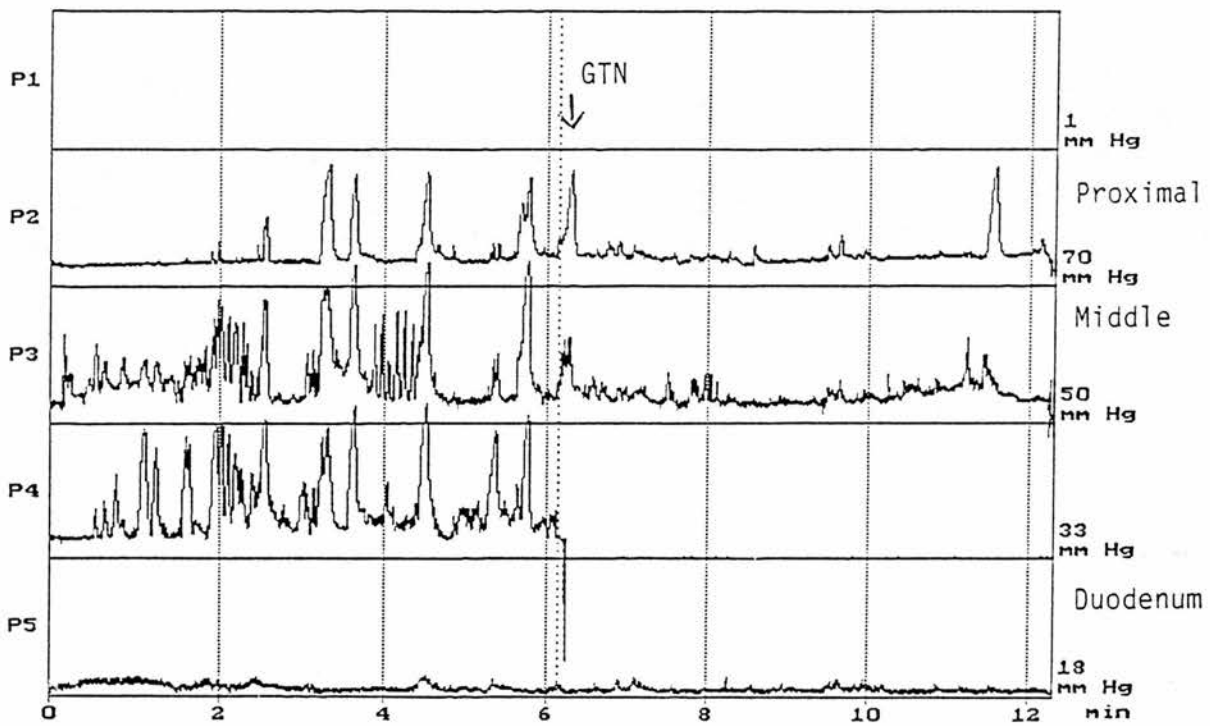


Figure 8.4. SO manometry tracing during infusion with GTN (condensed to paper speed of 1cm/min). There was abolition of phasic activity recorded at the proximal and middle channels after GTN infusion. The distal channel was disconnected and used as the infusion port.

CHAPTER NINE

INCIDENCE OF THE POST-LAPAROSCOPIC CHOLECYSTECTOMY SYNDROME

9.1 Introduction

Since its introduction in 1987, laparoscopic cholecystectomy (LC) has become the standard treatment for symptomatic gallstone disease. The advantages of LC over open surgery are well documented [382-384]. As discussed in chapter 4, no study has prospectively examined the post-cholecystectomy syndrome in relation to LC.

9.2 Patients and Methods

9.2.1 Patients' characteristics

100 consecutive patients admitted for LC between June 1994 to June 1995 were recruited into the study. The diagnosis of cholelithiasis was confirmed by ultrasonography in all cases. Patients were excluded if open cholecystectomy had been planned or they were unable to cooperate in answering the questionnaires. Indications for cholecystectomy and details of investigations performed prior to surgery were obtained from medical notes.

9.2.2 Questionnaires

The symptom profile of the patients was evaluated by a standard questionnaire administered by the author of this thesis (see appendix two) which examined characteristics of pain (site, duration, frequency, quality, periodicity, precipitating and relieving factors), other dyspeptic symptoms (nausea, vomiting, heartburn, food intolerance and early satiety) and colonic symptoms (bloating, constipation, diarrhoea). A previous history of hysterectomy and of psychiatric disturbances were also evaluated. Questionnaires were administered to patients before surgery and at least six months postoperatively. Patients were followed up in routine outpatient review or by telephone interview. Their notes were reviewed for operation details, postoperative morbidity, complications, and gallbladder histology.

LC was done as a four-trocar technique with electrocautery dissection by four consultant surgical teams. Intraoperative cholangiography was not routinely performed. Patients with a history of jaundice, abnormal liver function tests, dilated

common bile duct and pancreatitis underwent pre-operative ERCP and sphincterotomy.

9.2.3 Statistical methods

Logistic regression analysis was performed to develop a model to predict pain after LC. Simple comparisons of pre-operative symptoms between patients with post-LC pain and those with successful outcome were performed (chi-squared $p<0.05$). Significant factors (chi-squared $p<0.05$) were then entered into the logistic multiple regression to construct a classification table.

Analysis of the associated symptoms was subdivided into dyspeptic and colonic symptoms. Gallbladder histology was classified as normal, mild (mild chronic infiltrate confined to lamina propria), moderate and severe (changes of cholecystitis with severe acute and chronic inflammatory infiltrate, fibrosis and atrophy of gallbladder wall).

The studies were approved by the Lothian Ethical Subcommittee for Medicine and Oncology.

9.3 Results

9.3.1 Incidence of post-cholecystectomy pain

Three patients were excluded from the final analysis because the planned LC was converted to open surgery. One patient had an empyema of the gallbladder and two patients had dense adhesions. The remaining 97 patients were all successfully followed up. No patients were excluded from the study because they were unable to answer the questionnaire.

There were 19 males (20%) and 78 females (80%). The median age was 50.9 (range 19-85) years. There was one complication each of biliary leak and bleeding. Two other patients reported new onset of diarrhoea after LC. Thirteen patients (13%) reported pain similar to that present before surgery and were defined as the

'symptomatic group'. Patients who became pain free were defined as the 'asymptomatic group'. All of the patients with post-cholecystectomy pain had normal postoperative liver function tests and ultrasound scans; five patients had a normal ERCP. Two patients had *Helicobacter pylori* positive duodenal ulcer disease on gastroscopy. Symptomatic relief was obtained after eradication of the organism.

9.3.2 Preoperative characteristics

Indications for surgery and duration of symptoms are shown in table 9.1. Patients underwent LC because of either chronic biliary pain or complicated gallstone disease (acute cholecystitis, cholestatic jaundice or pancreatitis).

The majority of patients complained of upper abdominal pain (either epigastric or right hypochondrium) (table 9.2). The duration and frequency of pain, the quality and periodicity of pain were equally distributed in those who were asymptomatic and symptomatic after LC. The prevalence of preoperative dyspeptic and colonic symptoms is shown in table 9.3. The common dyspeptic symptoms were nausea (60%), food intolerance (54%) and heartburn (30%). The majority of patients noted fatty food intolerance. Of the colonic symptoms, bloating (50%) was the most common.

The thirteen patients who continued to complain of pain following LC differed in several respects from those who had a successful outcome. All but one of the thirteen patients had a history of chronic biliary pain, and only one underwent surgery because of acute cholecystitis ($p<0.05$, table 9.1). These patients had also been subjected to more pre-operative investigations (gastroscopy or barium enema) than those with a successful outcome ($p<0.05$, table 9.4). Previous hysterectomy was not commoner in those with post-cholecystectomy pain. The majority of patients who had a successful surgical outcome had histological evidence of moderate or severe cholecystitis (64%), whilst the group with persistent pain usually had normal histology or mild changes of cholecystitis (77%).

As shown in table 9.3 and 9.4, bloating ($p<0.001$), constipation ($p<0.05$) and previous or current consumption of antidepressant drugs ($p<0.001$) were significantly commoner in the poor outcome group after analysis with chi-square statistics. Psychiatric medications was defined as either current or previous medications with antidepressant or anxiolytic drugs. Eight patients were taking psychiatric drugs (6 on antidepressants, 2 on anxiolytics) at the time of surgery. However, logistic regression analysis showed that only bloating and consumption of antidepressant drugs were significantly associated with PC pain. With these two variables, a classification table (table 9.6) was produced which could predict the outcomes of pain free and persistent pain after surgery with 95% and 65% accuracy respectively

9.3.3 Postoperative characteristics

This is shown in table 9.5. Heartburn was not cured by LC although most patients did not feel that this was a failure of cholecystectomy as they did not expect heartburn to be relieved by surgery. Heartburn did not develop six months after cholecystectomy. The cure rates for nausea and early satiety were in excess of 80%. The reported incidence of nausea, vomiting and early satiety after surgery was largely confined to patients who continued to experience pain after surgery. The incidence of food intolerance was also low after LC but the majority of patients continued on low fat diet after surgery.

9.4 Discussion

The incidence of post-LC pain was 13%. Preoperative abdominal bloating and use of psychiatric drugs were significantly more common in these patients.

Studies that have assessed symptomatic outcome after open cholecystectomy have given varying results. Ros and Zambon evaluated 93 patients two years after cholecystectomy [308]. Only 53 patients were completely symptom free. Konsten et al [309] followed up over 300 Dutch patients through postal questionnaires to their general practitioners at a median interval of 10 years after cholecystectomy and reported symptoms in 18%. Gilliland and Traverso followed up over 600 patients for a mean

follow up period of 45 months and found that 88% of patients had complete symptomatic relief after cholecystectomy [311]. Patients with symptoms associated with gallstones such as bloating, nausea and vomiting, but without the pain of biliary colic were less likely (75%) to obtain symptom relief from cholecystectomy than those with biliary colic. Around 1% of patients reported occasional diarrhoea. Dyspepsia was the most common residual symptom after cholecystectomy, occurring in 32 percent.

The reported varying incidence of the post-cholecystectomy syndrome can be ascribed to the varying methods of evaluation, wording of questionnaires, and patient case mix. Abdominal pain, heartburn, nausea, flatulence and other dyspeptic symptoms are variously recorded and words such as dyspepsia and flatulence mean many different things to different patients.

There have been few studies which have examined symptoms following LC. In a 3-month follow-up of 52 patients, Peters et al reported that 77% of patients considered their symptoms to be cured by the procedure [312]. In a comparative study of open and LC, Vander Velpen et al reported that 95 percent of their patients thought that they had obtained overall symptomatic improvement [313]. Qureshi et al performed analysis of patients' perceptions of postoperative symptoms and global satisfaction [314]. They noted that 25% of patients complained of more than two symptoms postoperatively but 84% considered the procedure to be a complete success. In a study of an earlier cohort of patients from the same institution as the current study, Wilson and Macintyre evaluated symptomatic outcome of 115 patients a year after laparoscopic cholecystectomy and compared the outcome with 200 patients who had undergone the open procedure. Over 90% of patients in both groups considered the procedures to be successful in achieving symptomatic relief [315]. McMahon et al reported over 90% of patients were improved after the operation [385]. None of these studies assessed patients symptoms in prospective manner i.e. only patients' postoperative symptoms were evaluated and patients were either expected to list [314] or their case notes were reviewed for preoperative symptoms.

The incidence of post-cholecystectomy pain reported in this series is similar to that reported for open cholecystectomy [311]but is higher than that reported by Wilson and Macintyre [315]. These authors found that 94 percent of patients considered the operation to have cured or improved their symptoms and only 6.6 percent complained of having abdominal pain every day or most days following cholecystectomy. This may be because 34 percent of patients in their series had acute cholecystitis compared to 20% in the current series and this higher proportion may help to account for the better results [315].

Patients with atypical biliary pain usually underwent extensive upper gastrointestinal or colonic investigations before being referred for surgery. Despite this cautious approach, two patients with post-cholecystectomy pain were subsequently found to have *Helicobacter pylori* associated duodenal ulcer disease. It is noteworthy that only one patient with post-cholecystectomy pain arose from the group with complicated gallstone disease. Patients in the symptomatic group also underwent more pre-operative investigations than those in the asymptomatic group.

Ros and Zambon observed that patients with a shorter duration of symptoms before cholecystectomy achieved higher rate of symptom relief than those with longer preoperative history [308]. The present study did not find that symptom duration, quality and periodicity of the pain predicted outcome. This is consistent with the observations that the quality and periodicity of gallstone pain are variable [317-323]. In a study of patients admitted with acute upper abdominal pain, the pain characteristics did not accurately predict gallstones [324].

In contrast with the finding by Vander Velpen et al [313], symptomatic relief from heartburn or de novo development of this symptom after LC did not occur. It has been shown that after cholecystectomy, the constant flow of bile into duodenum induces duodenogastric reflux which is commoner in patients with dyspepsia than asymptomatic patients [386]. However, this finding is controversial and other

investigators have not confirmed this observation [387]. Incidence of food intolerance fell after surgery but majority of our patients continue on low fat diet. The cure rate for nausea, vomiting and early satiety is excellent as reported by Vander Velpen et al [293].

Pre-operative abdominal bloating and constipation were significantly more common in the symptomatic group. The combination of both symptoms is often present in patients with irritable bowel syndrome. Most interestingly, history of psychiatric disturbances was a significant predictor for post LC pain; and this has not been previously reported. McMahon et al also found that patients with poor outcome had higher anxiety and depression scores when they were assessed after surgery [385].

Patients with post LC pain had a higher prevalence of normal or minimally inflamed gallbladder. A tentative proposition is that the abdominal pain described by patients in this subgroup was unrelated to gallstones disease but was due to irritable bowel syndrome or non-ulcer dyspepsia. There may be a relationship between this often described as 'functional' disorder and major life events and depression [388,389]. Patients with non-ulcer dyspepsia are known to be more neurotic, more prone to anxiety and depression than controls [390,391]. On logistic regression analysis, only bloating and previous or current consumption of antidepressant were significantly associated with persistent pain. The model developed from these two variables could predict PC pain with 65% accuracy. This model looks encouraging though clearly needs further improvement. Future questionnaires should examine anxiety and depression level perhaps using the Hospital Anxiety and Depression questionnaire. Ultrasonographic findings such as number and size of stone; and thickness of gallbladder wall might prove to be significant variables.

The cholecystectomy rate has increased since the introduction of the laparoscopic approach [294]. This is probably due to a reduced surgical threshold. This study confirms the importance of defining symptoms which are due to gallstones rather than other gastrointestinal conditions. Surgical outcome is likely to be poor if it is

offered to difficult patients with dyspepsia who have undergone extensive investigations. Their dyspepsia is usually poorly relieved despite acid suppression therapy. Patients with dyspepsia and symptoms of irritable bowel symptoms i.e. bloating and constipation, and use of psychotrophic drugs are unlikely to benefit from LC.

Table 9.1 Indications for laparoscopic cholecystectomy and duration of symptoms.

Baseline characteristics	**number of patients (%)**	
	Asymptomatic	Symptomatic
	84	13
Indication		
Chronic biliary pain	54 (64%)	12 (92%)*
Complicated gallstone disease	30 (36%)	1 (8%)
Acute cholecystitis	20 (24%)	1 (8%)
Cholestatic jaundice	6 (7%)	0
Pancreatitis	4 (5%)	0
Duration of symptoms		
Less than 6 months	54 (64%)	10 (77%)
More than 6 months	30 (36%)	3 (23%)

* $p<0.05$

Table 9.2 Incidence of pain characteristics.

Pain characteristics	Number of patients (% in the group)		Frequency in study group (%)	p-value
	Asymptomatic	Symptomatic		
Site				
Upper abdomen	77(92%)	13(100%)	93	NS
Lower abdomen	7(8%)	0(0%)	7	NS
Radiation to back	49(58%)	11(85%)	60	NS
Quality				
Sharp	32(38%)	6(46%)	38	NS
Cramp	18(21%)	1(8%)	19	NS
Burning	6(7%)	2(15%)	8	NS
Crushing	4(5%)	1(8%)	5	NS
Dull	24(29%)	3(23%)	27	NS
Periodicity				
Constant	56(67%)	7(54%)	65	NS
Colic	28(33%)	6(46%)	35	NS
Duration (minutes)				
<30	50(59%)	9(69%)	61	NS
>30	34(41%)	4(31%)	39	NS
Frequency(per week)				
>1 attack	38(45%)	8(61%)	47	NS
<1 attack	46(55%)	5(39%)	53	NS

Table 9.3 Incidence of dyspeptic and colonic symptoms.

Symptoms	Number of patients (% in the group)		Frequency in study group (%)	p-value
	Asymptomatic	Symptomatic		
Dyspepsia				
Nausea	50(60%)	8(62%)	60	NS
Vomiting	28(33%)	3(23%)	32	NS
Heartburn	29(35%)	4(31%)	34	NS
Early satiety	14((17%)	4(31%)	18	NS
Food intolerance	44(52%)	8(31%)	54	NS
Colonic Symptoms				
Bloating	36(43%)	12(92%)	50	<0.001
Constipation	7 (8%)	10(77%)	11	<0.05
Diarrhoea	3(6%)	2(15%)	5	NS

Table 9.4 Incidence of abdominal surgery, psychiatric medications and histological findings.

	Number of patients		Frequency (%)	p-value
	Asymptomatic (84)	Symptomatic (13)		
Abdo. surgery				NS
No surgery	50(60%)	7(54%)	59	
Hysterectomy	8(10%)	1(8%)	9	
Other surgery	26(31%)	5(38%)	32	
Psychiatric medications	13(15%)	8(62%)	22	<0.001
Gallbladder Histology				<0.01
Normal/mild	30(36%)	10(77%)	41	
Moderate/severe	54(64%)	3(23%)	59	
Pre-operative Investigations				<0.05
Gastroscopy	1(1%)	5(39%)	17	
Barium enema	0	5(39%)	5	

Table 9.5 Prevalence of preoperative and postoperative symptoms.

Symptom	Number of patients		Cure rate in percentage*
	Preoperative	Postoperative	
Nausea	50	3	94
Vomiting	28	10	65
Heartburn	29	28	3
Early satiety	14	2	85
Food intolerance	44	9	80
Bloating	36	11	69
Constipation	7	7	0
Diarrhoea	5	7	0

*Cure rate is defined as the proportion of those with a given symptom preoperatively who had relief of symptom at six months.

Table 9.6 Classification table for prediction of post-operative pain

Predicted	**Actual**	
	Pain free	PC Pain
Pain free	80	5
PC Pain	4	8
Accuracy %	95	65

CHAPTER TEN

THE EFFECTS OF CHOLECYSTECTOMY UPON SPHINCTER OF ODDI MOTILITY

10.1 Introduction

Although cholecystectomy is generally regarded as a safe and successful treatment of symptomatic gallstone disease, a close postoperative follow-up reveals that between 20 and 50% of patients after cholecystectomy continue to have some abdominal pain [308,314,315]. Sphincter of Oddi (SO) dysfunction has been said to account for 16% of such patients [326].

The gallbladder and SO are closely related in function. In the filling phase of the gallbladder, the SO contracts while the gallbladder relaxes, resulting in influx of bile into the gallbladder. In its emptying phase the SO relaxes to permit an outflow of bile from the bile duct into the duodenum. It is possible that cholecystectomy alters biliary physiology, giving rise to SO dysfunction in some cases. The present study examined SO motility before and six months after laparoscopic cholecystectomy.

10.2 Patients and Methods

10.2.1 Patients selection

Seven female patients known to have symptomatic gallstone confirmed on ultrasonography were recruited into the study. They were all awaiting LC for their gallbladder disease. The exclusion criteria were stones in the common bile duct as suggested by cholestatic liver function test and ultrasound scan; history of pancreatitis; previous gastric surgery and vagotomy; medication with motility, anticholinergic or psychotropic drugs; presence of diabetes, neuropathy and severe cardiovascular and respiratory disease. All patients completed a symptom questionnaire, which detailed aspects of their presenting history, other abdominal symptoms and medication.

10.2.2 Biliary manometry

Biliary manometry was performed 2-3 weeks before and six months after laparoscopic cholecystectomy. After overnight fast, patients were sedated with 5-10 mg of midazolam intravenously. Biliary manometry was performed by the standard

water perfusion technique with triple-lumen polyethylene manometric catheter as described in section 5.3.

10.2.3 Analysis of manometric recordings

Manometric tracings were coded and analysed by an experienced observer who was blinded to the status of patients.

10.2.4 Statistical analysis

All data are reported as mean±SEM unless otherwise stated. Paired Student's tests were used to compare for changes in motility variables before and after LC. Baseline value was defined as the value obtained before stimulation with CCK.

The studies were approved by the Lothian Ethical Subcommittee for Medicine and Oncology.

10.3 Results

Five patients successfully completed the study and were considered for analysis. In one patient the bile duct could not be cannulated; one further patient declined to return for follow-up manometry after LC. The median age for the remaining five patient was 52 years (Range 38-61). The median duration of symptomatic gallstone disease was 6 months (Range 2-18). None of the patients who completed the study had features of irritable bowel syndrome as defined by the modified Rome criteria [392]. All of the patients underwent uncomplicated LC and reported excellent symptomatic relief after surgery . One patient developed mild pancreatitis after her repeat biliary manometry and had an uneventful recovery.

The results of the SO motility variables are shown in table 10.1. The mean common bile duct pressure was 5.5±3.0 before LC. The value remained unchanged after surgery. There was no significant change in the baseline motility variables after LC.

SO manometry was possible for approximately 2 minutes after CCK injection. Longer recordings were not possible because CCK appeared to induce retching and intolerance.

Before LC, CCK injection consistently reduced or abolished SO phasic activity with a small and insignificant reduction in basal pressure that remained around 24 mmHg (see figure 10.4 for typical SO manometry tracing after intravenous injection with CCK). After LC, CCK failed to reduce SO phasic activity. The duration of phasic activity was the only motility parameter which was significantly reduced by CCK after LC from 6.1±0.2seconds to 4.4±0.6 seconds. No significant reduction was noted in the amplitude and frequency of phasic waves.

The values for the amplitude, frequency, duration and motility index of CCK stimulated sphincteric phasic activity after LC were significantly elevated when they are compared with similar CCK stimulated values before surgery. The changes in amplitude, frequency and duration of sphincteric phasic activity for individual patients with CCK stimulation before and after surgery are shown graphically in figure 10.1 to 10.3. Before LC the motility index decreased from (491±96 mmHgsec) to (81±35 mmHgsec) after CCK. There was only a slight reduction in the motility index with CCK stimulation after LC (from 416±79 to 351±43 mmHgsec). The motility index following CCK stimulation after LC was significantly higher than that before surgery ($p<0.01$). A "paradoxical response" to CCK was not observed in any patient after LC.

10.4 Discussion

Six months after LC, the SO loses its inhibitory response to CCK. This finding has not been previously documented. Tanaka et al measured bile duct pressure before and 3 weeks after cholecystectomy using a microtransducer method [393]. They found that after cholecystectomy, SO spasm induced by morphine injection caused significantly higher bile duct pressure than before surgery. However, caerulein promptly reduced the pressure by relaxing the SO. Their finding suggests that the

gallbladder functions as a pressure reservoir during spasm of SO before cholecystectomy.

The variables of baseline SO motor activities i.e. the recordings obtained before CCK, are consistent with the values obtained by other workers using similar perfusion technique [346,350.353]. CCK injection frequently provoked agitation and restlessness although satisfactory recordings were obtained in all subjects for a minimum of two minutes. The reason behind the induction of agitation is unknown but CCK is known to provoke panic attacks in healthy volunteers [150]. Before LC, CCK injection induced relaxation of the SO. There was reduction of the amplitude, frequency and motility index in all the patients. In some patients, there was abolition of all phasic activity during the recording interval.

Our findings in man are analogous to those reported by Grace and Pitt in cats [394]. These investigators showed that basal SO motility was unaffected by cholecystectomy and that the sphincter was not relaxed by intraduodenal fat infusion or by an injection of CCK. It is also known that injection of tetrodotoxin or lignocaine into the cystic duct of animals prevented SO relaxation in response to gallbladder distension [67,69,70]. It is therefore likely that gallbladder and SO function are co-ordinated by nerve fibres which pass from the gallbladder to the SO and such fibres have been demonstrated by anatomical dissection [50,67,68].

We speculate that in man, as in experimental animals, post-cholecystectomy SO dysmotility in response to CCK is due to denervation which occurs as a consequence of cystic duct amputation.

The actions of CCK on the gallbladder and SO are known to be mediated by myogenic and neuronal receptors. In the gallbladder, CCK stimulates postganglionic cholinergic neurones and this action is blocked by atropine [11]. CCK also stimulates gallbladder smooth muscle directly, since the atropine resistant effect is unaltered by complete denervation with tetrodotoxin. CCK inhibits the SO by stimulating

postganglionic nonadrenergic noncholinergic inhibitory neurones, and this action is inhibited by tetrodotoxin but not atropine. CCK also directly contracts the SO by stimulating excitatory receptors upon the smooth muscle, and this action is not inhibited by tetrodotoxin [11]. Under physiological conditions it is likely that CCK has a neurally mediated inhibitory effect upon the SO that overrides the direct excitatory effect of the hormone upon the SO smooth muscle. These conflicting dual effects of CCK have been demonstrated in other experimental situations. For example, after truncal vagotomy in prairie dogs, there is an increase in baseline resistance to flow through the SO [62] and an increase in frequency and amplitude of phasic contractions of the SO [63].

Thus we speculate that following cholecystectomy, denervation leads to loss of the CCK mediated neural inhibitory effect of the SO with preservation of its direct excitatory effect.

The patients described in this study all had an excellent symptomatic response to cholecystectomy with complete abolition of pre-operative biliary type pain. We nevertheless speculate that our findings are relevant to the "post-cholecystectomy syndrome" of abdominal pain, disordered serum liver function tests, delayed bile duct emptying and abnormal SO manometry [377]. A range of SO motility profiles have been reported in such patients; fixed SO tonic hypertension is probably due to fibrosis occurring as a consequence of common bile duct surgery. A range of phasic motor pattern disturbances including "tachyoddia" and a paradoxical response to CCK [327,328] are more difficult to explain, but our findings may be relevant to such observations.

Disordered SO motility may lead to elevation of the bile duct pressure due to loss of the pressure reservoir effect of the gallbladder [393] and this could give rise to biliary pain in some patients. We have shown that disordered SO motility in response to CCK can be a consequence of cholecystectomy and it is not inconceivable that an exaggeration of this could cause pain in susceptible individuals with irritable bowel

syndrome thus leading to the post-cholecystectomy syndrome. These patients are known to have reduced pain threshold to distension throughout the gut [395-397].

Table 10.1 Sphincter of Oddi motility variables (Mean+SEM) before and after laparoscopic cholecystectomy.

	Before LC	**After LC**
Mean CBD pressure (mmHg)	5.5±3	6.8±2.3
Baseline		
SO pressure (mmHg)	24±7	27±3
SO frequency (per minute)	5.2±0.6	4.4±0.8
SO amplitude (mmHg)	94±11	101±17
Duration (second)	5.7±0.8	6.1±0.2
Motility Index (mmHgsec)	491±96	416±79
After CCK		
SO pressure (mmHg)	22.6±6	26±4
SO frequency (per minute)	1.6±0.7	4±0.7*
SO amplitude (mmHg)	30.5±12.5	94±11*
Duration(second)	1.9±0.8	4.4±0.6*
Motility Index (mmHgsec)	81±35	351±43*

LC means laparoscopic cholecystectomy.

* p value is <0.05 . This is for comparison of variables with CCK injection before and after laparoscopic cholecystectomy.

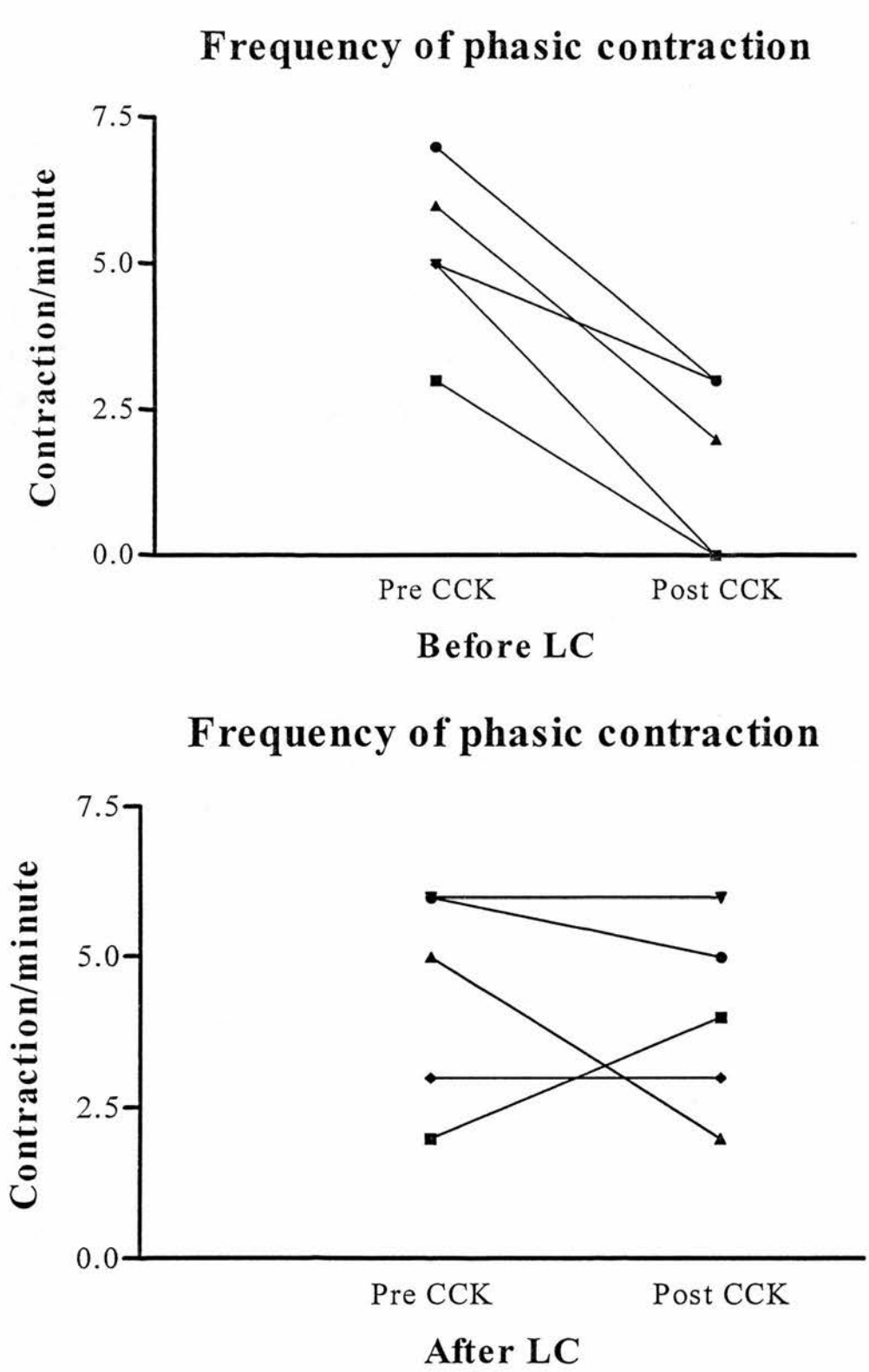
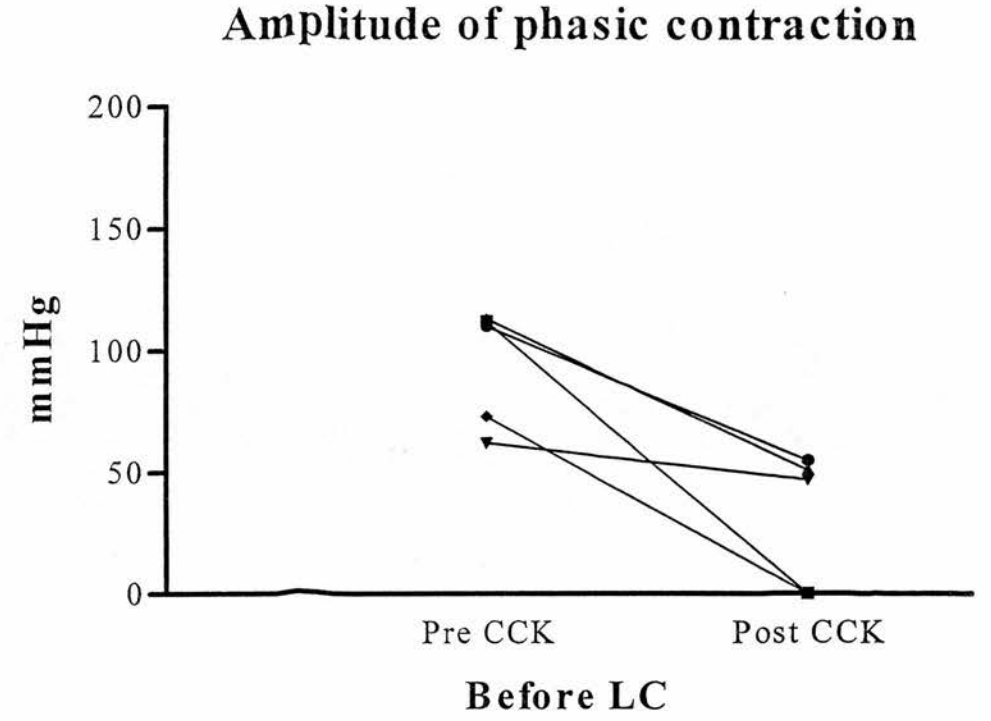


Figure 10.1 Frequency of phasic contractions with CCK administration before and after laparoscopic cholecystectomy.



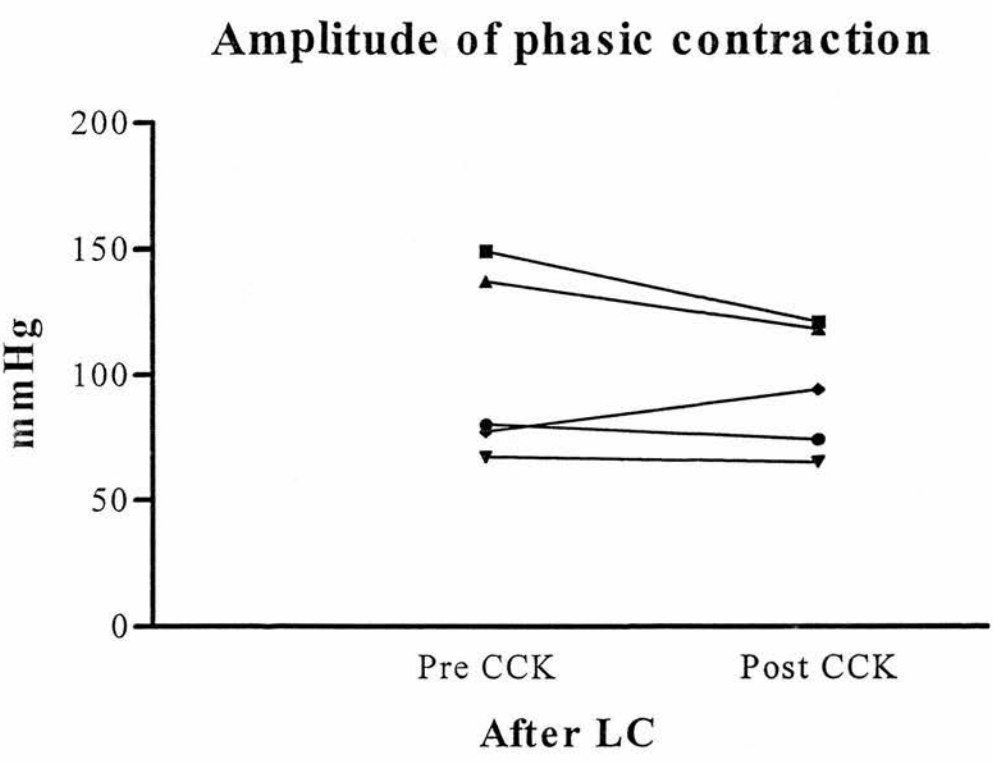
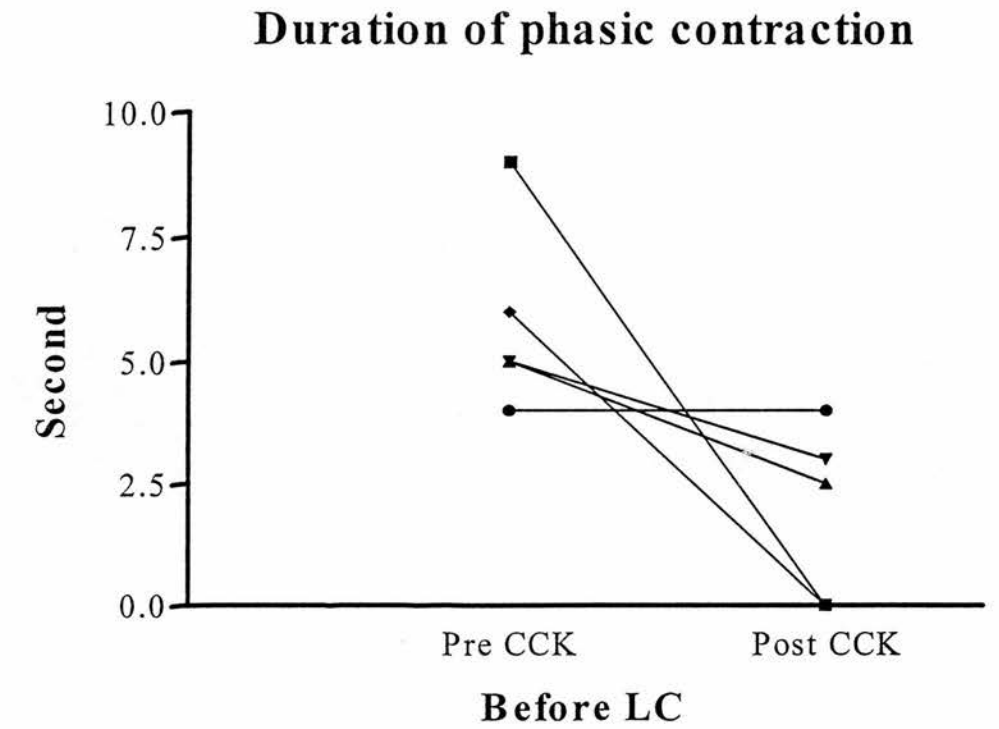


Figure 10.2 Amplitude of phasic contractions with CCK administration before and after laparoscopic cholecystectomy.



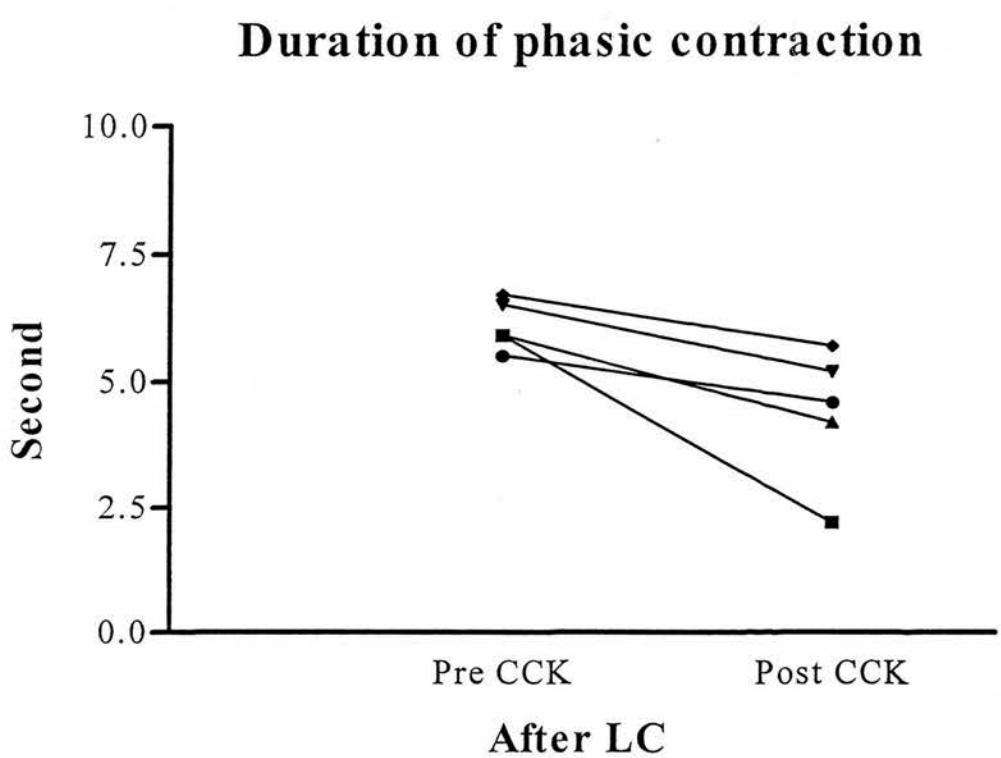


Figure 10.3 Duration of phasic contractions with CCK administration before and after laparoscopic cholecystectomy.

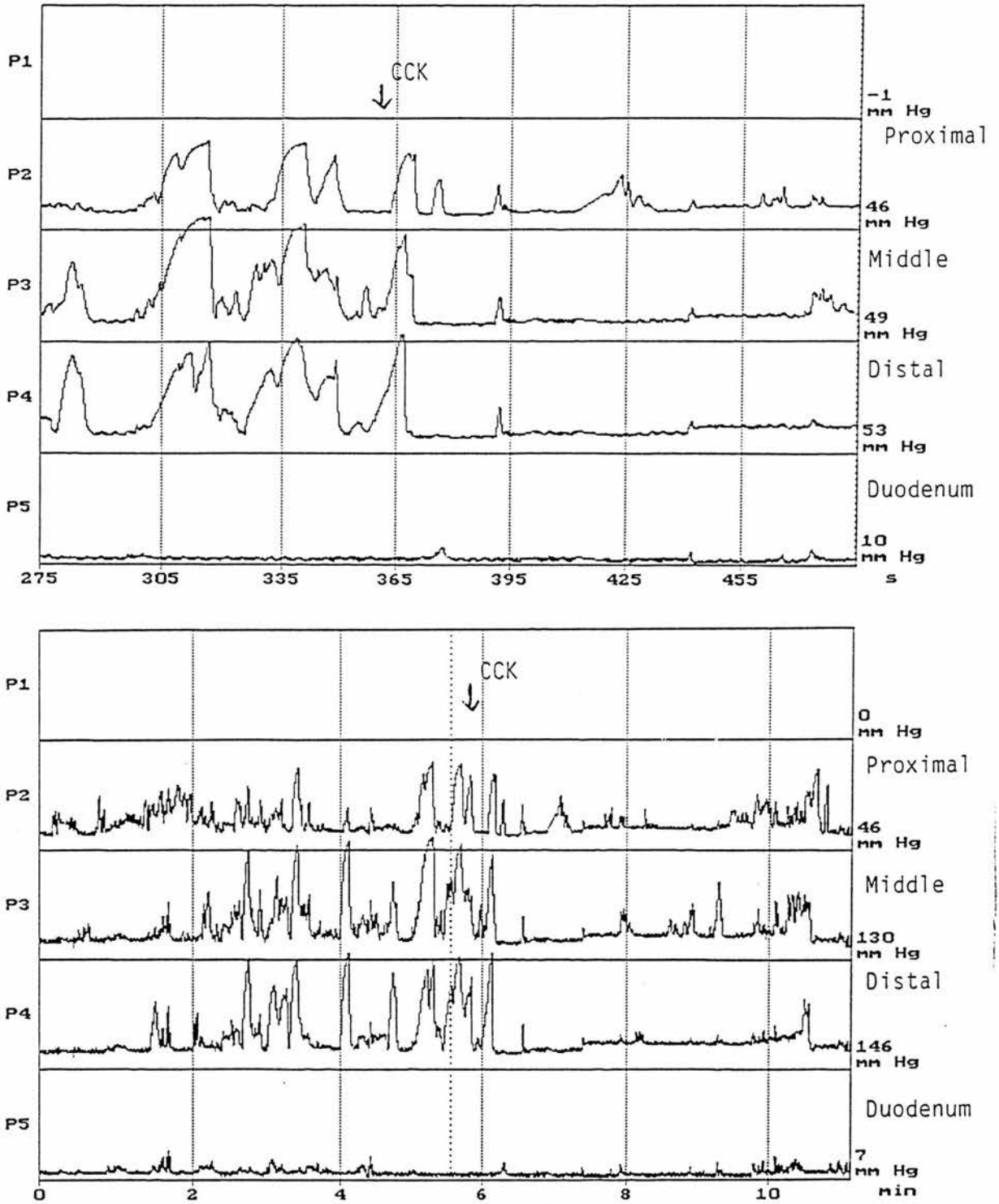


Figure 10.4 SO manometry tracing before and after intravenous injection of CCK showing reduction of phasic contractions (top chart at paper speed of 4 cm/min and bottom chart condensed to 1 cm/min) .

PART THREE

SUMMARY AND CONCLUSIONS

CHAPTER ELEVEN

SUMMARY, CONCLUSIONS AND THOUGHTS FOR FUTURE

11.1 Introduction

My work described and discussed in this thesis examined several physiological aspects of biliary motility and changes in SO motility which occur after LC.

In this chapter, I would like to summarise the findings and conclusions reached from each project, and finally to discuss the ideas and concepts I have developed.

11.2 Early gallbladder emptying

I confirmed that gallbladder emptying preceded gastric emptying of solid food. It was demonstrated that this early phase of gallbladder emptying was accompanied by increased plasma CCK and gastrin concentrations. Gastrin is unlikely to be responsible for gallbladder contraction from previous investigators' observation. Neither gaseous gastric distension nor sham feeding consistently stimulate gallbladder emptying or release of CCK and gastrin. In a minority of subjects, sham feeding stimulated significant gallbladder emptying which was accompanied by increase of plasma immunoreactive CCK concentration. CCK could therefore be responsible for the early phase of gallbladder emptying. The mechanism underlying the early release of CCK is unclear but it could be stimulated by vagal cholinergic mechanism during the cephalic phase. Alternatively during the gastric phase, intragastric nutrients could stimulate the release of bombesin which is known to stimulate CCK release in humans.

It is also possible that the early release of CCK could be due to entry of small amounts of swallowed food into duodenum causing CCK release before the detection of labelled solid particles in the intestine. This issue can be resolved if the threshold for CCK release by intraduodenal nutrient stimuli is known.

11.3 NO and gallbladder emptying

Systemic infusion of the NO donors GTN and SNP impaired post-prandial gallbladder emptying. This inhibitory effect was not secondary to hypotension or changes in post-prandial release of CCK. Direct inhibition by NO of gallbladder smooth muscle contraction was confirmed *in vitro* using isolated bovine and human gallbladder muscle

strips. However, negative immunohistochemical staining for neuronal NOS in gallbladder tissues suggested that although NO directly inhibits gallbladder smooth muscle contraction, it is unlikely to be the neurotransmitter for NANC innervation regulating gallbladder motility. The evidence noted in this thesis failed to demonstrate a physiological role for NOS in gallbladder motility.

11.4 Nitric oxide and the sphincter of Oddi

Topical application of GTN inhibited SO motility and duodenal contraction. 5 mg and 10 mg were found to be equally effective.

11.5 The incidence of post-laparoscopic cholecystectomy syndrome

Symptoms were prospectively assessed using standard questionnaire before and six months after LC. Thirteen percent of patients had continuing symptoms after LC. Abdominal bloating and consumption of antidepressant before LC were found to be significantly associated with post-cholecystectomy pain. With these two variables, a classification table was produced which could predict the outcomes of pain free and persistent pain after surgery with 95% and 65% accuracy respectively.

11.6 Effects of laparoscopic cholecystectomy upon sphincter of Oddi motility

SO dysfunction may be a consequence of cholecystectomy and could lead to biliary symptoms following surgery. In health the gallbladder and SO function are co-ordinated by nervous and hormonal pathways. I examined the hypothesis that cholecystectomy interfered with normal SO function by disrupting the controlling pathways. SO manometry was performed in five women, a few days before and six months after LC which was undertaken for uncomplicated cholelithiasis. Before LC, an injection of CCK largely inhibited phasic SO activity and tended to reduce tonic pressure. Following surgery, there was a loss of the inhibitory response of the SO to CCK. The findings may be of relevant to the range of manometric abnormalities noted in patients with SO dysfunction.

11.7 Thoughts for future work

(1) It is possible that the early release of CCK and gallbladder emptying so induced was due to entry of small amounts of nutrients into duodenum stimulating CCK release before detection of labelled solid particles in the intestine. However, it would be difficult to design an experiment in which intragastric nutrients could be prevented from entering duodenum in order to examine the role of cephalic or gastric phase in isolation during early gallbladder emptying. This would involve surgical dissection with disruption of the gastro-biliary nervous pathways in experimental animals. Theoretically, balloons could be distended in the pylorus to prevent the flow of nutrients into duodenum but this technique is uncomfortable and does not ensure complete blockage of gastric fluid flowing around the balloon into duodenum. We feel that this issue can be resolved by examining the threshold of intraduodenal nutrients for CCK release. This can be performed by naso-duodenal intubation with fine bore catheter. Amino acids and fatty acids of varying concentrations can then be instilled into the upper part of small bowel with simultaneous measurement of plasma CCK concentration. If the threshold is found to be high, then it would be unlikely that the entry of small amount of nutrients into the duodenum is responsible for CCK release and early gallbladder emptying.

The role of vagal cholinergic stimulation of CCK release and direct stimulation of gallbladder smooth muscle contraction can be examined by giving a group of volunteers cholinergic blockage with atropine. Gallbladder emptying and CCK release can then be examined after ingestion of test meals.

(2) It is possible that impaired gallbladder motility in chronic cholecystitis is due to excessive local production of NO by submucosal inflammatory cells. A future study could examine diseased gallbladder muscle strips, including assessment of NOS and *in vitro* response to L-NMMA.

(3) Topical application of GTN on the ampulla could facilitate cannulation of the ampulla at ERCP. This may facilitate extraction of small to medium size bile duct

stones without the need for either sphincterotomy or sphincteroplasty. These hypotheses can be tested in randomised study examining ease of ductal cannulation and stone extraction by endoscopists of similar experience with and without GTN application.

(4) The model developed in the thesis was predictive for persistent pain after LC with only 65% accuracy. Its specificity and sensitivity could be improved with inclusion other variables and the model needs to be tested prospectively. Future questionnaires could examine anxiety and depression level using validated tool such as the Hospital Anxiety and Depression questionnaire. Ultrasonographic findings such as number and size of stone; and thickness of gallbladder wall might prove to be significant variables.

(5) The clinical relevance of the loss of inhibitory response of SO to CCK following cholecystectomy is unclear. None of our patients suffered from post-cholecystectomy pain. It would be of interest to confirm this finding in a larger population to establish whether there is any regaining of the inhibitory response with time after LC and whether the response differs in asymptomatic and symptomatic individuals.

It is hoped that this work and the concepts and ideas developed will inspire and link with other projects by future members of the research group working in this area.

APPENDIX ONE

Publications and presentations to learned societies

Some of the work of this thesis has been presented to learned societies and published in either abstract or full form.

Presentations to learned societies

W Luman, GD Smith, WG Haynes, DJ Webb, KR Palmer.
Nitric oxide may influence postprandial gallbladder motility; in vivo and in vitro study.
(Presented at Caledonian Society of Gastroenterology Autumn Meeting 1995).

W Luman, KR Palmer.
Sphincter of Oddi and cholecystectomy.
(Presented at Edinburgh Surgical Research Meeting, Royal College of Edinburgh, Jan 1996).

W Luman, JEF Ardill, E Armstrong, GD Smith, WG Haynes, DJ Webb, KR Palmer.
Nitric oxide and abnormal gallbladder function - do gut hormones have a role ?
(Presented at the British Society of Gastroenterology Spring Meeting 1996).

W Luman, S Ghosh, GD Smith, JEF Ardill, E Armstrong, KR Palmer.
Early gallbladder emptying is mediated by cholecystokinin.
(Presented at the British Society of Gastroenterology Spring Meeting 1996).

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Incidence of post-cholecystectomy pain after laparoscopic cholecystectomy.
(Presented at the American Digestive Disease Week 1996).

W Luman, S Ghosh, GD Smith, JEF Ardill, E Armstrong, KR Palmer
Early gallbladder emptying - Intestinal or central?.
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Publications

W Luman, GD Smith, WG Haynes, DJ Webb, KR Palmer.
Nitric oxide may influence gallbladder motility.
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The post laparoscopic cholecystectomy syndrome - a prospective study.
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W Luman, AJK Williams, A Pryde, GD Smith, SJ Nixon, RC Heading, KR Palmer.
The effect of cholecystectomy upon sphincter of Oddi motility.
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W Luman, A Pryde, GD Smith, RC Heading, KR Palmer.
Effect of topical glyceryltrinitrate upon sphincter of Oddi motility.
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Manuscripts submitted

W Luman, S Ghosh, GD Smith, JEF Ardill, E Armstrong, KR Palmer.
Mechanisms of early gallbladder emptying.
(submitted to Gut)

W Luman, JEF Ardill, E Armstrong, GD Smith, L Brett, AM Lessells, WG Haynes, DJ Webb, KR Palmer.

Nitric oxide and gallbladder motor function.

(submitted to Gut)

APPENDIX TWO

Questionnaire for cholecystectomy symptoms

(see attached sheets)

CHOLECYSTECTOMY QUESTIONNAIRE

Name:

Date of birth:

Age:

Date of cholecystectomy:

Date of questionnaire:

Indication for surgery:

1) Chronic abdominal pain
2) Pancreatitis
3) Jaundice
4) Acute cholecystitis

Duration of symptom:

1) Less than 6 months
2) More than 6 months

Have you experienced any further pain after cholecystectomy?

1) Yes
2) No

Is the pain similar to preoperative pain?

1) Yes
2) No

Where is the site of pain?

1) Upper abdomen
2) Lower abdomen

Duration of pain

1) Less than 30 minutes

2) More than 30 minutes

Frequency of pain

1) More than one attack per week

2) less than one attack per week

How would you describe the character of the pain?

1) Crampy

2) Dull

3) Sharp and stabbing

4) Crushing

5) Burning

How would you describe the nature of the pain?

1) Colicky

2) Constant

Does the pain radiate to the back?

1) Yes

2) No

What brings on the pain?

1) Food

2) Stress

3) Nothing

Do you have other abdominal symptoms?

1) Constipation

2) Diarrhoea

3) Bloating

Is the pain associated with any other features below?

1) Nausea
2) Vomiting
3) Heartburn
4) Early satiety
5) Food intolerance

Have you been on antidepressant/anxiety medication, or seen a psychiatrist before?

1) Yes
2) No

Are you taking an antidepressant?

1) Yes
2) No

Have you had other surgery before?

1) Gastric surgery
2) Hysterectomy
3) Other

Medications:

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