Animal model for training in sentinel lymph node biopsy of the stomach through combined methods[1]

Modelo animal para treinamento em pesquisa de linfonodo sentinela em estômago mediante métodos combinados

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ABSTRACT
PURPOSE: Create and validate a proposed animal model for training in sentinel lymph node biopsy of the stomach.
METHODS: In thirty-two rabbits, through a laparotomy, they received a subserosal injection of 0.1 ml of phytate labeled with technetium-99m (0.2 mCi) in the anterior wall of the gastric corpus, followed by 0.2 ml of Blue Patent V® 2.5%, through the same puncture site. Suspicious lymph nodes were searched *in vivo* at five, ten and 20 minutes, both visually (Blue Patent stained lymph nodes) and with a manual *gamma* radiation detector (to detect suspected radioactive lymph nodes). After 20 minutes, was performed resection of these for further evaluation of radioactivity (*ex vivo*) and histological study.
RESULTS: Lymph nodes were identified in 30 rabbits (Average of 2.2 lymph nodes per animal). Of the 90 suspected lymph nodes that occurred in the study, 70 cases (77.8%) were histologically confirmed for lymphoid tissue. Of these, the majority were located in the periesophageal region of the gastric fundus. The sample presented a mortality rate of 6.25% and nine complications related to the method, which interfered in the identification of the lymph nodes.
CONCLUSION: The animal model for sentinel node biopsy in rabbit stomachs proved to be feasible, with low complexity and reproduced the difficulties encountered for gastric lymph node biopsy in humans, being adequate for surgical training.
Key words: Models, Animal. Sentinel Lymph Node Biopsy. Stomach. Coloring Agents. Technetium. Rabbits.

RESUMO
OBJETIVO: Criar e validar uma proposta de modelo animal para o treinamento em pesquisa de linfonodos sentinelas no estômago.
MÉTODOS: Em trinta e dois coelhos, mediante laparotomia, foi injetado na subserosa da parede anterior do corpo gástrico, 0,1 ml de fitato marcado com tecnécio-99m (0,2 mCi), seguido pelo mesmo orifício, de 0,2 ml de Azul Patente V® 2,5%. A cavidade abdominal foi avaliada, *in vivo*, por meio de inspeção para pesquisa de suspeitas de linfonodos azuis e com detector manual de radiação *gamma* aos cinco, dez e 20 minutos para pesquisa de suspeitas de linfonodos radioativas. Após 20 minutos, foi realizada a ressecção dessas para posterior avaliação da radioactividade (*ex vivo*) e estudo histológico.
RESULTADOS: Foram identificados linfonodos em 30 coelhos (Média de 2,2 linfonodos por animal). Das 90 suspeitas de linfonodos ocorridas no estudo, em 70 casos (77,8%) obteve-se confirmação histológica para tecido linfóide. Destas, a maioria estava localizada na região periesofágica do fundo gástrico. A amostra apresentou taxa de mortalidade de 6,25% e nove intercorrências relacionadas ao método, que interferiram na identificação dos linfonodos.
CONCLUSÃO: O modelo animal para pesquisa de linfonodos sentinelas em estômago de coelhos mostrou-se factível, pouco complexo e reproduziu as dificuldades encontradas para a pesquisa de linfonodos gástricos em humanos, sendo adequado para o treinamento cirúrgico.
Descritores: Modelos Animais. Biópsia de Linfonodo Sentinela. Estômago. Corantes. Tecnécio. Coelhos.

Introduction

Through studies carried out by Gould *et al*.[1], Cabanas[2], Morton *et al*.[3], we have the basis of the concept of sentinel lymph node biopsy (SLNB).

After the consolidation of SLNB in cancers of the skin[3-5] and breast[5-7], lymphadenectomies related to surgeries for the treatment of these neoplasms have become selective, and are no longer routine, resulting in a decrease in morbidity and mortality rates. In the last decade, SLNB has also been carried out for cases of early gastric cancer[5,8-27].

The initiatives to perform SLNB in early gastric cancer are justified by the less invasive procedures (smaller gastrectomies and the use of videolaparoscopic access), less extensive lymphadenecomies (D1), with consequent preservation of gastric function, better esthetic appearance, less morbidity and mortality, and better quality of life after surgery, without jeopardizing the oncological safety of the treatment and survival of these patients[19,24,28].

However, the stomach has characteristics that make SLNB difficult, such as the existence of a multi-directional lymphatic drainage system, the presence of skip metastasis[9-11] and an average identification of sentinel nodes of more than two per individual[5,9,16,12-15].

Fundamental, following the studies of Claude Bernard (1865), the Nuremberg Cod (1947) and the Helsinki Declaration (1975), it is advisable that experiments on human beings only be carried out based on evidence from research on animals[29-31].

There have been only a few publications related to the creation of an experimental animal model for SLNB of the stomach, using experiments on swine[32], dogs[33] and rabbits[34]. However, they have limitations, such as their small sample size[9,11], high cost, and large sentimental humanistic appeal, especially when dogs are involved. Also, for the establishment of an experimental model, it is necessary to take into consideration the characteristics of the animal and its similarities to humans[29-31,35].

Even after careful review of the literature, based on the databases: Medline; Lilacs; ScieLo; Cochrane BVS, no anatomical description of the gastric lymph node in rabbits could be found[36], and the same applies to its comparison with humans.

All that is found is the description of the dimensions of the rabbit stomach, which are similar to that of a human infant[37], and a description of the gastric wall of the animal, which is thinner, especially in the regions of the body and fundus[35,37,38], with the occurrence of stratification of this gastric wall in layers (submucosal, muscle proper and serosa)[37-39], consisting of a three-dimensional lymphatic plexus that interconnects them, without any apparent differences in terms of their distribution in the stomach[36].

This study aims to develop an appropriate experimental animal model for training in gastric lymph node biopsy, through the use of a radiolabeled colloid (RC), associated with the handling of hand-held gamma probe.

Methods

Thirty-two healthy, male rabbits (*Oryctolagus cuniculus*) of the New Zealand breed, aged between 3.0 and 5.5 months (average 4.6 months) with body weights of between 3000 and 4200 grams (average 3407.8 grams), were submitted to intramuscular anesthesia with ketamine hydrochloride (30 mg/Kg) and xyaline hydrochloride (3 mg/Kg), and local infiltration of the abdominal wall with 10 ml of 1% lidocaine.

The animals were then tied in position, in decubitus dorsal, on the surgical table.

Lymph node biopsy

Following middle xiphotransumbilical laparotomy, the presence of radioactivity was evaluated inside the abdominal cavity, using a hand-held *gamma* probe (Europrobe©), i.e. the initial background (BG).

Next, the subserosa of the gastric wall was injected at point X (**PX**), using a 26-guage needle, 0.1 ml of RC consisting of phytate colloid marked with radioisotope tecnécio-99m at 0.2 mCi, followed by 2 ml of 2,5% Patent Blue dye *Guerbet*® (AzP), injected by the same orifice, pressing lightly on the site for 5 minutes. PX was determined on the anterior wall of the gastric body, 3.5 cm from the small curvature in the region between the two anterior branches of the left gastric artery.

For the biopsies, a needle was introduced 0.5 cm into the subserosal layer (Figure 1).
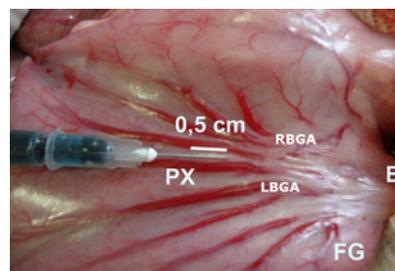

FIGURE 1 - Injection (with AzP) in Point X (**PX**), in the subserosa of the gastric wall. **E.** Esophagus; **FG.** Gastric Fundus; **LBGA.** Anterior left branch of the left gastric artery; **RBGA.** Anterior right branch of the left gastric artery.

After five, ten and 20 minutes of RC and AzP injection, the *"in vivo"* evaluation of radioactivity inside the abdominal cavity was repeated, using the hand-held gamma probe (*gamma probe*), to look for suspect lymph nodes (SL).

The *"in vivo"* evaluation was carried out by visual identification of the lymphatic ducts originating from the injection and possible lymph nodes stained by AzP, as well as through evaluation, with a gamma probe, of the presence of radioactivity in these points, known as SL (Figure 2).
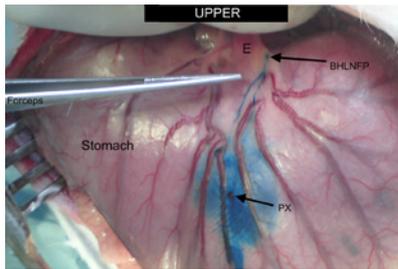

FIGURE 2 - Evaluation of the abdominal cavity at ten minutes, demonstrating the presence of a suspected blue-radioactive lymph node (**BHLNFP**), subsequently confirmed histologically as lymphoid tissue. In the photo we have a pincer pointing to a lymphatic duct stained in blue. **E.** Esophagus; **PX.** Point X; **BHLNFP.** Blue-hot lymph node gastric-fundic periesophageal.

The SL were classified as: blue only (dyed and monitored for inferior radioactivity ten times that of the initial or final BG); radioactive only (not dyed, but with radioactivity count higher than ten times that of the initial or final BG, and radioactive blue (both conditions present). They were then sub classified by anatomical localization (Table 1). Observation: The final BG consists of the radioactivity count emitted by the interior of the abdominal cavity after exeresis of the organs.

After the last evaluation at 20 min with the gamma *probe*, dissection of the SL was performed, associated with total gastrectomy and splenectomy, according to the standard technique[35].

Euthanasia was performed by increasing the anesthesia with 25 mg/ml Sodium Thiopental overdose and subsequent injection with 10 ml of 19.1% potassium chloride.

Finally, the radioactivity presented by the same structures as those evaluated *in vivo* was evaluated *ex vivo,* along with the gastric content.

Observation: The purchase cost of the animals was recorded.

Histological confirmation study

The resected SL, duly identified, were fixed in 10%

formaline buffer for at least 12 hours. They were then embedded in paraffin, cut to a thickness of 6 μm and stained with hematoxilin-eosin, to confirm the presence of absence of lymphoid tissue.

This study is part of a project approved by the Ethics Committee on the use of Animals of the State University of Campinas – SP, under protocol n°. 2033-1.

Statistical analysis

The variables of analysis were compared, using the statistical tool ANOVA for repeated measurements.

The transformation by ranks was applied to the means, due to the variably, and for comparison of these the Wilcoxon test was used for related samples.

To determine the agreement between the methods, the interclass correlation coefficient (ICC) was used[40].

The level of statistical significance adopted in this study was $p < 0.05$.

Results

In 32 animals studied through the combined SLNB methods, 90 SL were identified, obtaining histological confirmation of lymphoid tissue in 70 cases (Table 1).

TABLE 1 - Distribution of 70 lymph nodes by location and classification according to SLNB method.

LYMPH NODE LOCATION	Blue only	Radioactive Only	Blue-Radioactive	TOTAL (%)
Group of perigastric lymph nodes:				
Between the stomach and the gastric fundus	1	1	21	**23 (32.86%)**
Along the small curvature	6	0	7	**13 (18.57%)**
Periesophageal next to the small gastric curvature	4	1	7	**12 (17.14%)**
Anterior to the gastric fundus	1	2	2	**5 (7.14%)**
Anterior to the gastric fundus	2	0	3	**5 (7.14%)**
Group of lymph nodes further from the stomach:				
Next to the vena cava	3	0	3	**6 (8.57%)**
Next to the pancreas	0	0	4	**4 (5.72%)**
Retroesophageal	0	0	2	**2 (2.86%)**
	17	**4**	**49**	**70 (100%)**

Note: No SL were found along the greater curvature of the stomach.
Lymph nodes were identified in 30 animals (93.75%), with an average of 2.2 per animal (Table 2).

TABLE 2 - Results of sentinel node detection according to the method used.

Variables	LN	detection	method
	Dye	Isotope	Combination
Nº. animals in who LN were identified (%)	30 (93.75%)	27 (84.37%)	30 (93.75%)
Average of the Nº. of LN identified by An (SD+/-)	2.06 (1.39)	1.66 (1.31)	2.19 (1.35)
Sensitivity (%)	66/70 (94.3%)	53/70 (75.7%)	70/70 (100%)
Positive predictive value (%)	66/86 (76.7%)	53/66 (80.3%)	70/90 (77.7%)
Accuracy (%)	66/90 (73.3%)	60/90 (66.6%)	70/90 (77.7%)*

Note: Nº. = Number of; LN = Lymph nodes; % = percentage, SD+/- = Standard deviation; An = Animal. *If only lymph nodes identified in the 10 minute evaluation are considered, we would have accuracy of the combined method of 40/54 (74.07%).

Of the 90 SL, 10 cases presented histological confirmation for more than one lymph node, i.e. in seven cases for the same suspicion, there was histological identification of two associated lymph nodes and in three cases, three associated lymph nodes were identified.

Two deaths occurred in the study (6.25%). Of these, one occurred during the evaluation of the abdominal cavity at 10 minutes by overdose of anesthetics, and the other during dissection of a suspected paracaval lymph node by vascular lesion (Table 3). During the study, there were 20 complications, 14% of which made SLNB unfeasible (Table 3).

Applying ICC to demonstrate the level of agreement between the suspicion generated by the SLNB methods and histological confirmation, for the evaluations carried out at 5, 10 and 20 minutes, higher levels of agreement were found for the radioactive only SL up to 10 minutes (ICC = 0.552, with IC95% = 0.188 - 0.770) and blue only or blue-radioactive, at 20 minutes (ICC = 0.524, with IC95% = 0.147 - 0.772). In relation to the two cases in which no lymph nodes were identified, one was in an animal that had suffered an accident due to loss of RC and AzP, which had spilled into the abdominal cavity, and the other case was related to the death that was caused by anesthetic overdose.

The sizes of the lymph nodes ranged from 0.3 to 6.0 mm (mean = 2.05 mm and SD +/- 1.30).

The mean radioactivity observed for the radioactive lymph nodes *in vivo* at times five, ten and 20 minutes was 170.7 cps (SD+/- 181.7), 174.6 cps (SD+/-204,5) and 254.7 (SD+/- 384.7), respectively. In the evaluation *ex vivo,* they presented mean radioactivity of 221.5 cps (SD+/- 241.2).

As for the evaluation of radioactivity presented by the first lymph node identified, there was a statistically significant increase in this value over time, during evaluation *in vivo* at five (p = 0.0004) and ten minutes (p = 0.0023) when compared with the evaluation at 20 minutes.

The majority of the SL (60%) and lymph nodes (57.1%) were identified in the evaluation with the gamma probe *in vivo* up to 10 min.

The mean radioactivity of the gastric content related to the animals with absence of perforation of the gastric wall was

TABLE 3 - Intercorrences during the study, according to time of experiment.

INTERCORRENCES	During Anesthesia	LP	Puncture of the RC AzP	5 min	10 min	Dissection LN
DID NOT INLUENCE THE LYMPH NODE BIOPSY						
Animal cried out*	1					
Lesion of the sorosa of the gastric wall*		1				
Pneumothorax		2				
Small spread of the RC/AzP at the puncture site			5			
Leakage of teh RC/AzP after pressing the site				1		
Small hemorrhage during dissection of suspected paracaval lymph node						1
INFLUENCED THE LYMPH NODE BIOPSY A LITTLE						
Perforation of the gastric wall**			3			
Major spreading of the RC/AzP at the puncture site			3			
JEPORDIZED THE LYMPH NODE BIOPSY						
Death					1	1
Loss of RC/AzP with contamination of the abdominal Wall			1			
TOTAL	1	3	12	1	1	2

Note: LP = During laparotomy; RC/AzP = Radiolabeled colloid and Patent Blue; LN = Lymph nodes; Min, minutes. *These occur in the same animal, and are related to causality, since the cries generate gastric distension, favoring the lesion of this organ during laparotomy; **In these three cases, gastric perforation was demonstrated after checking the mean quantity of radioactivity by the gastric content, respectively, 616 cps, 460 cps and 220 cps; cps, Number of counts per second of radcioactivity confirmed by the gamma probe (Europrobe©) in five to ten seconds[5].

2.15 cps (SD +/- 2.45). The mean value exhibited by the initial BG of the whole sample was zero cps and that of the final BG was 6.87 cps (SD+/-15.01), ranging from 0 to 79.5.

The cost of purchase of the animals was R$12.00/Kg.

Discussion

There have been initiatives that use SLNB in humans for gastric cancer aimed to indicating more economic lymphadenectomies and gastric resections[15,18,19,24,25], and this conduct is currently becoming accepted in global clinical practice[9,11,26], especially after the recommendation of the Japanese multicenter trial on the use of SLNB in early gastric cancer (T1 or T2, N0, M0)[27].

In the current study, even knowing that this animal model presented a stomach without neoplasia, this did not invalidate the objective of the study, since its aim was to demonstrate the complexity of anarchic gastric lymphatic drainage, showing the possibility of appropriate treatment for the identification of lymphatic ducts and lymph nodes in varied topographies, as well as the acquisition of the necessary skills for gastric wall biopsies and appropriate handling of the gamma probe.

In fact there are already specialists around the world carrying out SLNB on humans[11,18,19,24,25,27,28], who can serve as sources for learning the technique, yet the presence of a feasible animal model would avoid possible ethical problems related to the training of new professionals, as well as the occurrence of errors related to the learning curve that could jeopardize the safety of the oncological treatment.

In this model, knowing that the learning curve for the use of SLNB is estimated in 30 cases[9,11], we considered it advisable to submit the professional executing this study, a trained surgeon, with no previous experience of the methods, to an equivalent sample size number, to adequately demonstrate its difficulties.

Despite the existence of other experimental animal models for SLNB in the stomach, in dogs[33], pigs[32] and rabbits[34], the model presented in this study had the following main advantages: low purchase cost of the animals, ease of handling, absence of the need for preoperative fasting, high animal replacement capacity (especially due to the short reproductive cycle), good adaptation to the laboratory environment, and limited humanistic sentimental appeal[30,31,35] (Chart 1). Moreover, it was the first national attempt that used rabbits[32-34], presented an acceptable mortality rate and practicality in the execution of the exclusively intramuscular anesthesia, without the need for definitive intubation[35].

Regarding the method chosen in this study, we used combined methods for SLNB[5,9,18,20,21], through injection of RC and staining in the subserosa of the gastric wall[13,23,26].

Due to the presence of a connection and free passage of the lymph of the lymphatic drainage system of the gastric wall, from the subserosal to the submucosal plexus, the injection site did not interfere in the results of the SLNB[16]. Furthermore, puncture in the subserosal region of the stomach wall is more practical, less technical, and faster to execute, and is ideal for use in individuals in training[16].

Due to the absence of a similar theoretical basis, the choice of the injection point (PX) was based on the need for practicality in terms of access to and execution of the SLNB, as well as inferences obtained through the results achieved in a previous pilot study, involving nine rabbits, in which the researchers injected dye and RC at three points of the gastric body, and in which where PX was the point that presented identification of a higher number of lymph nodes.

The SLNB in this animal model presented a detection rate and average number of lymph nodes found per animal that were comparable with other studies on humans[5,9,15,20,21].

It must be emphasized that the accuracy of the methods was influenced by the occurrence of 11% of SL that presented histological confirmation for more than one lymph node. Moreover, the sensitivity values presented in relation to the SLNB methods must be interpreted with care, since there was no neoplastic lesion in the present model.

In addition, due to the theoretical presence of lymph nodes in all the animals, it was not practicable to calculate the negative predictive value and specificity related to the methods.

However, these data revealed that the method with use of staining is more sensitive in this animal model, and supplements the evaluation carried out with the gamma probe[5,21].

In this experiment the identification and classification of the blue and/or radioactive lymph node occurred just before and/or during exeresis of the organs, although this procedure is recommended before the gastrectomy, at varied times, according to the characteristics of the dye used[5,10,12,15-17,21-23].

Nevertheless, the ideal time for evaluation in this model, based on the characteristics of the dye[5,9,11,14,22,23], anatomy[35] and dimensions of the lymphatic drainage system of the animal's stomach[36], should probably be ten minutes after the injection of AzP.

It can also be noted that the evaluation carried out at five minutes did not present a statistically significant difference for the identification of lymph nodes by the methods used, when

CHART 1 - Comparison of studies for the creation and standardization of animal experiments for SLNB of the stomach.

AUTHORS CARACTERISTICS	Méndez *et al.* 2003[32]	Kim *et al.* 2008[34]	Torres de Melo 2010[33]	Alves *et al.* 2012 (This study)
Animal	Swine	rabbits	dogs (female)	Rabbits (male)
Sample (N)	4	7	25	32
Method of SLNB	Combined	Combined	Combined	Combined
Site of puncture	Submucosa (local ND)	Body subserosa 5 cm from the small gastric curve	Anthro seromuscular in the small gastric curvature, 1 cm from the pylorus	Body subseroso at 3.5 cm of the small gastric curve
Substances injected (quantity in ml)	DTPA with AD (0.1ml 7.4 MBq) + 1% isosulfan blue (0.1ml)	NFM (0.1 ml) and 1% isosulfan blue (0.1ml)	99m Tc with phytate (0.2 ml/19 MBq) and patent blue 2.5% (0.2ml)	99m Tc with phytate (0.1ml/7,4 MBq) + patent blue 2.5% (0.2ml)
Puncture at the same point	Single	Separated / in sequence	Separated / > 20 min between punctures	separated / in sequence
Evaluation time of suspect lymph nodes	5-10 min	3 – 4.2 min*	0, 5, 10, 15, 20 and 40 min**	5, 10 and 20 min
Lymphadenectomy time	5-10 min	ND	after 40 min	after 20 min
Method of identification of lymphatic tissue	None	Histological study Hematoxylin-eosin	None	Histological study Hematoxylin-eosin
Identification of LN in the sample radiolabelled colloid/ dye (%)	ND	ND Combined methods = 100%	80% / 96%	84.37% / 93.5% (Combined methods = 93.75%)
Total Nº. of LN identified	8 LN radiativo e 6 LN azul***	****	27 radioactive LN + 39 blue LN*****	70 LN (combined methods)
Mean Nº. of LN per animal	ND	****	1.08 radioactive / 1.56 blue	2.19 lymph nodes/animal (combined methods)
Most frequent location of LN (frequency %)	Celiac region	ND	SL of the small curvature and infrapylorics	LN of the region between the esophagus and the gastric fundus
Nº. of false positives	NPC*****	0 % (ND)	NPC*****	22.22%
Accuracy	NPC*****	100%	NPC*****	77.77%

Note: LN = lymph nodes; DTPA = Diethylenetriaminepentaacetic acid; AD = Aminodextran; MBq = Mega Becquerel; 99m Tc = Technetium - 99m; ND = not determined; MFN = Magnetic fluorescent nanoparticles; NPC = Not possible to calculate due to lack of histological confirmation of the lymphod tissue; * = They did not establish time, only waited for the lymph nodes to turn blue or fluorescent; ** = Evaluations carried out over the times presented; *** = Criteria not defined to consider lymph nodes radioactive; **** = Cannot be evidenced due to absence of discription contained in the abstract and remainder of article written in Japanese (by symbols); ***** = Lymph nodes not histologically confirmed.

compared with the histological confirmation (staining only method – p = 0.7813; gamma probe evaluation only method – p = 1.0000; combined methods – p = 1.0000).

Furthermore, the evaluation carried out at 20 minutes with combined methods or AzP only was the condition that exhibited the highest concordance with the histological study (ICC = 0.524). (Exception: evaluations carried out up to ten minutes with the gamma probe, ICC = 0.552).

The 19 cases of false positive results occurred due to complications during the experiment, the most common of which occurred during the gastric wall punch and injection of RC and AzP (Table 3).

However, despite the complications, comparable to those that occurred in other studies conducted with human beings[5,9,21], with the help of the radio-guided evaluation using hand-held gamma probe, lymph node identification proved possible in most of the cases (93.75%).

The final BG (mean = 4.04 cps; SD +/- 5.9) did not

interfere in the SLNB[9].

It was ascertained that the lymph nodes presented mean dimensions below 2x those found in human beings[15]. However, this did not impair the validation of this model, since the macroscopic aspect of the lymph nodes should not be used as a criterion for determining the presence of metastatic invasion, as already demonstrated by the occurrence of 69% of metastases in lymph nodes measuring less than 5 mm[9,17].

Moreover, it is worth emphasizing that in two animals, it was suspected that three structures could be lymph nodes, even though they were not stained and radioactive. Two of these were lymph nodes (one located between the esophagus and gastric fundus and the other alongside the small curvature).

Conclusion

Animal model in rabbits is feasible, presents low cost in relation to the purchase of the animals, and low complexity, serving as the basis for the ethical execution of future studies related to the development and trial of new techniques, dyes and radiolabeled colloids for SLNB in the stomach. It also enables adequate training for professionals, particularly surgeons intending to carry out the procedure.

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