Chronic stress influence in panic development during SCUBA-diving practice: a pre-clinical study

Degree final project

November, 2019

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1. ABBREVIATIONS

ACTH: adrenocorticotropic hormone

AQC: acquisition task

AGE: arterial gas embolism

BLA: basolateral amygdala

CeA: central nuclei of the amygdala

CEEA: ethics committee of animal experimentation

CUS: chronic unpredictable stress

CRH: Corticotropin-Releasing Hormone

CRFR: corticotropin-releasing factor receptor

DAN: Divers Alerts Network

DCS: decompression sickness

DG: dentate gyrus

HPA: Hypothalamic-pituitary-adrenal

IMLCFC: Institut de Medicina Legal i Ciències Forenses de Catalunya

MeA: medial nuclei of the amygdala

MAO-A: monoamine oxidase A

PVN: paraventricular nuclei of the hypothalamus

PFC: prefrontal cortex

PBt: pulmonary barotrauma

PBt/AGE: arterial gas embolism following pulmonary barotrauma

qPCR: quantitative polymerase chain reaction

RT: retention

SCUBA: self-contained underwater breathing apparatus

UB: Universitat de Barcelona

UdG: Universitat de Girona

UWT: underwater trauma

2. ABSTRACT

Background: The investigation of SCUBA-diving fatalities is performed in a multidisciplinary team to elucidate the cause of death and the sequence of events that led to the fatality. This is performed determining: triggering factor, disabling agent, disabling injury and cause of death. Panic is reported as one of the most common triggering factors (40-60%) specially in arterial gas embolism following pulmonary barotrauma (PBt/AGE) deaths that performed a rapid ascend even in experienced divers. Chronic stress has been observed as a risk factor on the development of panic and therefore loss of control when a hazardous or unpredicted situation appears. This situation can lead to a reactive maladaptive behaviour and the diver, even knowing that it can be lethal, performs a rapid ascent.

Objective: To determine if chronic-stress impairs decision-making behaviour through a SCUBA-diving-like model, assessing rats behaviour after an underwater threatening situation and determining stress-related biomarkers (MAOA and CHRH1) in the prefrontal cortex and amygdala.

Methodology: the preclinical study has three phases. First, rats will be trained to perform a SCUBA-diving test, consisting of an acquisition task (ACQ) followed by a retention (RT) test. In the second phase rats will be divided in two groups and one of them will be exposed to chronic unpredictable stress (CUS) protocol. Finally, in the third phase, both groups will be exposed to SCUBA-diving test under threatening situation (water trauma; UWT) to assess their under-water choice-making behaviour. At the end, qPCR will be used to assess MAOA and CHRH1 mRNA expression as biomarkers of underwater impaired choice-making behaviour.

3. INTRODUCTION

3.1. Epidemiology

Diving practice is experimenting an uprising worldwide, approximately 7 million divers are active and 500 000 more are training every year. (1) Nowadays, the most common breathing method used in diving activities is SCUBA (self-contained underwater breathing apparatus) (1)

Although diving is a relatively safe sport there is an exposition to some physiological risks for example due to the change of pressure in the underwater that can lead to fatal outcomes (2). In every vertical descent of 10 metres there is an addition of a further 1.0 bar of pressure to the sea level (3).

There are few epidemiological reports about death in diving. In the last Divers Alert Network Report (DAN report) from USA and Canada from 2018 it's said that probably there were fewer than two deaths per million recreational scuba divers during 2016 (4), another report from Australia exposed that between 2002 and 2006 there was a fatality rate of 0.57 per 10^5 dives(5). In an European level there is data from the United Kingdom, over a 5-year period, from 1992 to 1996, where the mortality was observed to be 2.9 per 10^5 divers. (6)

In Spain, the exact incidence of these mortal accidents is not known but in Girona, every year there is about 1 death for each 100.000 scuba-diving immersions. (7)

3.2. Diving fatalities

The three most common pathological causes of death are drowning, arterial gas embolism and cardiac incidents. (2,8)

In most diving-related fatalities, between 52 to 86%, the identified cause of death is drowning. (9) but this conclusion may be incomplete, as it is essential to identify the sequence of events during the diving that led to the fatal outcome of drowning. (10)

The arterial gas embolism (AGE) is the second most common cause identified in about 13-24% (11,12) of SCUBA diving fatalities. This accident is usually due to a rapid ascent where the diver retains an excess of air, producing a lung overexpansion and an increase of pression in the thorax. If in this moment the diver experiences panic or there are some obstructions present in the airway it increases the difficulty to exhale the air. Then, the gas looks for natural

exits opening virtual spaces and alveolo-capillary and arteriovenous circuits producing the AGE. (7)

The third cause, cardiac incidents, in most cases is related to the medical comorbidity of the diver that is usually discovered during the autopsy. Most frequent medical comorbidities are coronary artery disease, cardiac arrythmias, and high blood pressure. (13)

Others causes of death that are also frequently reported are: trauma, decompression sickness that is included with AGE in the group of decompression illness, unexplained loss of consciousness and inappropriate gas that could be due to intoxication or a mistake in the mix of the gas (for example hypoxic gas mix). (2)

Except for an increase in deaths attributed to cardiac disease in the aging diver population, factors contributing to a fatal diving accident have not altered greatly over the decades. (14)

3.3. Sequential analysis

Some studies have proved that it is important related to diving fatalities not only to determine the cause of death but also to examine the underlying problems, factors and sequence of events that made the victims susceptible to the fatal incident. Those sequential events are defined as triggering factors, disabling agents, disabling injuries, and cause of death (13).

The triggering factor is considered as the earliest identifiable event that appeared to transform an unremarkable dive into an emergency. Some of the most common triggers are: exertion, panic that can also be considered as a disabling agent in some cases, buoyancy problems, disorientation and confusion (10). In the DAN report, the most common triggers were underlying health problem or equipment malfunctions. These problems that are not lethal, could cause panic on the diver and then it could interfere in their performance underwater and lead to the death.

The disabling agent is a dangerous behaviour or circumstance that is temporally or logically associated with the trigger and perhaps caused the disabling injury. For example, if there is a situation that provoked panic (trigger) to the diver, he responds doing a rapid ascent that would be the disabling agent. Other disabling agents are cardiac incidents, insufficient breathing gas and entrapment. (13)

Panic was reported as a disabling agent in one-fifth of the cases of emergency ascent (13) that could be produced for instance by a buoyancy problem (trigger) that would led to hyperventilation, anxiety and finally panic. (10)

The disabling injury is the direct responsible of the death or is the incapacitation that caused death due to drowning. Asphyxia is the most common disabling injury. (2)

The cause of death is the cause specified by the medical examiner. As mentioned before, the most common causes of death are drowning, arterial gas embolism, natural causes or internal diseases. In some publications, the cause of death is considered the same as the disabling injury or the direct consequence of the disabling injury. For example, drowning as a cause of death is secondary to loss of consciousness or an acute cardiac event. (2)

Triggering factor	→	Disabling agent	→	Disabling injury	→	Cause of death
Panic		Emergency ascent		Lung overexpansion		AGE
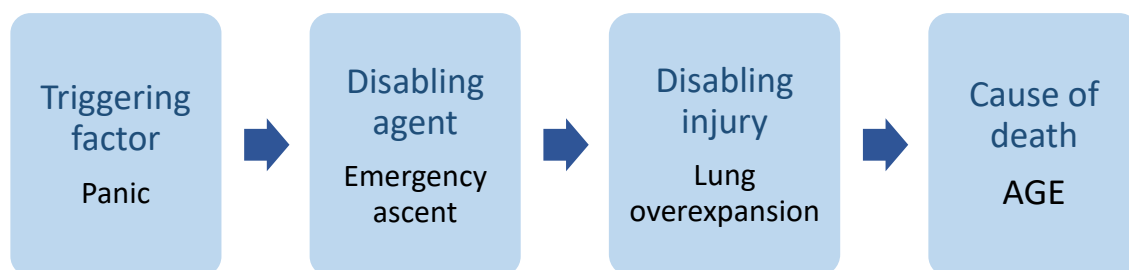
Figure 1. Paradigmatic sequence of events involved in diving fatalities. Adapted from (13)

3.4. Multidisciplinary investigation

The underwater media represents a difficulty in the investigation because of its conditions that are not present in the surface as the changes in pressure, density, the composition of the fluid and the flows(7).

This investigation is conducted with the goal to try to identify not only the cause of death but also the underlying problems, factors and the sequence of events, that made the victims susceptible to the fatal incidents(13). For this reason and its complex situation, diving related deaths must be investigated by a multidisciplinary team including the specialist police on underwater activities and the forensic pathologist.

The first step of the investigation is the police technical file. This file is used to record all the information of the investigation starting with the research, planification, trace and rescue of the corps. (7)

3.4.1. Research phase

When a diver disappears, the police starts the research recompiling information about the possible location from witness, companions and also the dive computer of the other divers. This information will be used to plan the research taking also into account different parameters like technical and environmental (7).

3.4.2 Technical-police inspection (7) (annex 1)

It starts when the corps is identified and consist on:

- Ocular subaquatic inspection of the corps and of the crime scene.
- Elaboration of a photographic inform of the location, position and state of the body and of the diving equipment(3).
- Sample taking and preservation of biological rests following the protocols of the custody chain.

Other important information that has to be recorded in the technical reports are data about the (7,14):

- Location of the corps: ubication, deep, and final position.
- Environmental situation: visibility, temperature, currents, marine animal activity...
- Final location of the diving equipment and collection of water samples.

When the rescue phase is concluded some exams must be performed (7,14):

- Testing of diving equipment. It is usually performed by dive experts from the police or equipment technicians to determine if it meets the legal standards.
- Gas analysis is performed by specialist laboratories to determine if it fulfilled the regulations. It is especially useful to discard carbon monoxide and other dangerous gas.

3.4.3 Dive profile

The dive computer is a device that nowadays most divers wear and can give important details of the fatal dive. It records information called the dive profile that informs about the depth, dive duration, ascent rates, the number of ascents, decompression staging and decompression stress, dive profiles (reverse or forward), water temperature, gas pressures, and gas consumption.
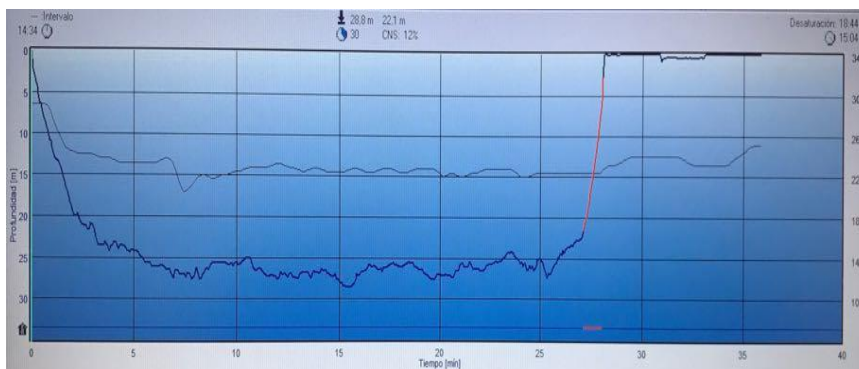
Figure 2. Dive profile showing a rapid ascent. Author: Fernando Aguirre, GEAS de la Guardia Civil. L'estartit (Girona)

This information allows an assessment of the likelihood of contributing factors such as pulmonary barotrauma (PBt) or decompression sickness (DCS) if it detects a rapid ascent, aspiration, gas toxicity, etc. (14)

When the dive profile is recovered it should be reviewed with independent diving experts to   determine all this information and it will also be useful to assess the diver's compliance with diving regulations. (3)

3.4.4   Autopsy

The autopsy is performed in three steps (15):

- o   Corps lift
- o   External exam
- o   Internal exam with the complementary tests: radiological, histopathological and toxicological (7).

- **Corps lift**

When the corps is found as integrand of the judicial commission or authorised by the Instruction magistrate, the forensic pathologist on duty will perform the corps lift. As all deaths in the water, the ones that are produced during the practice of SCUBA are considered of a violent nature and in some circumstances criminality suspicious (7).

The corps lift will be performed where it was discovered or where the boat that moved the corps docked. During the corps lift it is necessary to determine some aspects to stably the diagnostic of certain death, the death time and the moment of the death. If the death is in the hospital the clinical history has to be revised. (7)

- **Autopsy** (annex 2)

In the autopsy it is important to determine certain death, identify the deceased, differentiate between natural or violent typology, determine if the origin is homicidal, suicidal or accidental, know the cause of death and determine the circumstances of death (15) with the four steps of the sequential analysis: triggering factor, disabling agent, disabling injury and finally the cause of death. The autopsy should be performed in the first 24 hours after the death to avoid putrefaction artefacts. (7)

A macroscopic study is performed with an external and internal examination of the body using specific autopsy techniques and complementary exams like radiology, histopathological analyses, and toxicological results. (7)

Simple radiology of thorax and abdomen is the most common used to detect air due to its widespread use (11), but if the post-mortem computerized tomography is available, its use is recommended to detect the site and volume of abnormal gas spaces in the body prior to the autopsy as its non-invasive and very useful specially in cases of suspected AGE. (14)

Modifications in autopsy techniques allows the demonstration of gas in atypical sites, such as the cardiac chambers, vessels, pleura, peritoneum, and in other tissues. These techniques are difficult to perform but can allow the aspiration of the gas and analysis of its composition. Its analysis could indicate its source and become diagnostic. For example, gas from PBt usually reflects the inspired gas that is normal air or a helium/nitrogen/oxygen mixture. (14)

The techniques developed to demonstrate abnormal gas in the diving autopsy were designed to detect the gas embolism induced by PBt and DCS. Unfortunately, in some situations there is presence of gas from processes that are not related from diving or hyperbaric exposure and difficult its interpretation. These include post-mortem decompression artefacts, drowning, putrefaction, trauma, and resuscitation artefacts. (14)

Specially in the situations where gas is detected but its origin isn't clear, the dive profile must always be kept in mind; conclusions derived from the autopsy and complementary tests must appropriately fit the dive profile. Inadequate or incorrect conclusions are often based on the autopsy findings without the information available from the diving technicians, investigators, and physicians. (14)

3.5. Pulmonary barotrauma: diagnostic criteria

During the diving ascent, the compressed gas in the lungs expands with the falling ambient pressure. If intrapulmonary gas is prevented from escaping as a result of a closed glottis, bronchospasm or gas trapping, excessive transpulmonary pressures will result. Differential alveolar compliance results in differential expansion of adjacent lung units causing focal shearing between vessels and airways and rupture of small airways and/or alveoli. Escaping gas can enter the pleural cavity (pneumothorax), mediastinum (mediastinal emphysema that may extend down into the retroperitoneum or appear around the neck as subcutaneous emphysema), pericardial cavity (pneumopericardium) or pulmonary venules (which transits the left heart to result in arterial gas embolism). (1)

Gas bubbles entering the systemic circulation are distributed by blood flow. The cerebral circulation and specially the middle cerebral artery are common sites of bubble distribution. Intravascular bubbles trigger an acute inflammatory response with haematological and vascular components, which may result in reduced cerebral blood flow and finally infarction if there is an occlusion of the vessels, decreased integrity of the blood–brain barrier and cerebral oedema. Arterial gas embolism presents within seconds to minutes after a diver arrives to the surface, with profound neurological symptoms including paralysis, convulsions and coma, often accompanied by cardiovascular instability or cardiac arrest. (1)

Lawrence and Cooke have developed diagnosis criteria of the PBt/AGE to stablish the necessary supporting evidence for the diagnosis. (11)

Table 1. *Major and minor criteria for arterial gas embolism following pulmonary barotrauma (PBt/AGE)*

	Major criteria
1	History of a rapid ascent followed by loss of consciousness.
2	Gas in the left side of the heart, circle of Willis, coronary and retinal arteries.
3	Low probability of off gassing or decomposition.
4	Mediastinal or subcutaneous emphysema limited to the peri-thoracic area (e.g., supra-clavicular area) and/or pneumothorax.
	Minor criteria
5	Low air or panic situation.
6	Students or novice divers.
7	Over-inflated Buoyancy jacket or ditched weight belt
8	Dive computer evidence of a rapid ascent.
9	Other evidence of barotrauma, subcutaneous emphysema or pneumothorax.

Note: adapted from (11)

The diagnosis of PBt/AGE should probably only be made in the presence of a history of a rapid ascent and a rapid loss of consciousness and cardiac arrest, after surfacing or during ascent, which is unresponsive to resuscitation. (11) (criteria 1)

Rapid ascend can be proved with the diving profile obtained during the police investigation (criteria 8). The interpretation of the whole dive profile, post dive exposure and death interval is required (14) to determine if PBt/AGE is the probable cause of death.

The presence of gas in the body (criteria 2, 4 and 9) can be observed with the combination of well documented ante mortem situations, a uniform series of radiographs obtained within hours of death all interpreted by a single experienced radiologist and an autopsy performed by one experienced pathologist. (16)

To exclude off-gassing (criteria 3) is important to recognise that the diver was unresponsive before ascent and that pulmonary barotrauma and cerebral arterial gas embolism (PBt/AGE) was not the underlying problem. The presence of gas in this case is mainly from inert gas, previously breathed by the diver and then dissolved in the blood and tissues. Due to post-mortem decompression, usually during the ascendant of the corps in the rescue phase, the bubbles appear (14). This is the reason why it is important that a qualified team perform the rescue to reduce the effect of the artefacts. To avoid decomposition artefacts, if it is possible, the autopsy should be performed within 24h after death.

Nowadays, presence of a panic situation (criteria 5), although it is a common experience can only be confirmed with subjective statements of the people that witnessed the event and sometimes it can be imprecise or limited. (17)

3.6. Panic in SCUBA-diving fatalities

Panic is an extreme and inappropriate stress response to a real or imagined threat characterised by excessive anxiety where behavioural control becomes lost. (18). An expected result of a diver having a panic attack would be a rapid ascent or another flight response. (19)

Panic has been described as one of the most common triggering factors in diving fatalities being described between 40-60% of the deaths (9). In a US study, panic was identified as the disabling agent in one fifth of the cases of an emergency ascend (13). In a survey performed in the UK, 82% of the cases panic or anxiety was also identified as a triggering factor of a rapid ascent, being the rapid ascent the second-largest category of incidents(20).

Panic behaviour represents one of the most significant problems confronting divers of all ages and both sexes. Panic is not restricted to beginning divers; 54% of a sample of experienced divers have experienced panic or near-panic behaviour(21).

The developing of a panic situation depends on the individual factors and partly due to the skill level of the diver. Any event within a situation has the potential to trigger panic if it is a stressor for that specific individual. The variability in previous experiences, perceptions and skill sets that what is a stressful event for one diver may not be an event to another. (22)

Individuals naturally vary in their ability to regulate emotions and tolerate extremes of physiological arousal. There is evidence that individuals who are characterised by elevated levels of trait-anxiety are more likely to have panic behaviour as a response when exposed to stressors(21,22). Apart from trait anxiety, external factors like having the appropriate equipment, being informed and aware, and be competent in relevant skills, are very important to respond effectively to a situation (22).

If a diver encounters a problem underwater, lack of competence and therefore perceived inability to enact a solution, are psychological stressors that lead to increased physiological arousal. When arousal levels exceed tolerable levels and a person is unable to think clearly enough to find an effective solution, then there is an increasing risk that instinct behavioural responses appear (fight/flight) and those lead to reactive behaviour that is maladaptive for the scuba diving context (22). For example, trying to reach the surface excluding other vital factors, such as exhaling during ascent (18).

These changes in cognitive function and particularly executive function (planning and problem solving ability) that reduce the ability to find a solution are due to limbic system activation resulting in changes within the neural connections to the pre-frontal cortex (23).

3.7. Limbic system functions during stress

During the experience of stress, the organism initiates a generalized endocrine, autonomic, and behavioural response, which together increase the ability of an organism to withstand the physiologic and psychological threats encountered in life (24).

A stress response, created by a real or perceived threat (stressor), can be defined as an emergency state of an organism in response to a challenge to its homeostasis. (25)

The limbic system (hippocampus, PFC and amygdala) has significant effects on HPA axis regulation. In contrast to the hippocampus and the prefrontal cortex, the amygdala is thought to activate the HPA axis (26). The medial (MeA) and central (CeA) nuclei of the amygdala respond to different stressors (27). Neurons in the MeA are activated following exposure to "emotional" stressors including predator, forced swim, social interaction, and restraint stress paradigms (26,28). In contrast, the CeA appears to be preferentially activated by "physiological" stressors, including haemorrhage and immune challenge (29,30).

The prefrontal cortex (PFC) intelligently regulates our thoughts, actions and emotions through extensive connections with other brain regions.

Under stress conditions, the amygdala projections to the brainstem and to the hypothalamus, activates stress pathways (23) and stimulates the release of catecholamines and glucocorticoids throughout the neuroaxis. This excessive production of catecholamines can be due to an increase of the neurons firing in response to aversive stimuli. (31)

High levels of catecholamines and glucocorticoids strengthen amygdala and hippocampus functions, fear conditioning and consolidation of emotionally relevant information (32,33), but weaken PFC abilities such as working memory and attention regulation.

To sum up, these high levels of catecholamine release initiated by the amygdala during stress switch the brain from thoughtful, reflective regulation by the PFC to more rapid reflexive regulation by the amygdala and other subcortical structures. These mechanisms might save our life when we are in danger and need to react rapidly, but they can be detrimental when we need to make choices that require thoughtful analysis and inhibitory control. (23)
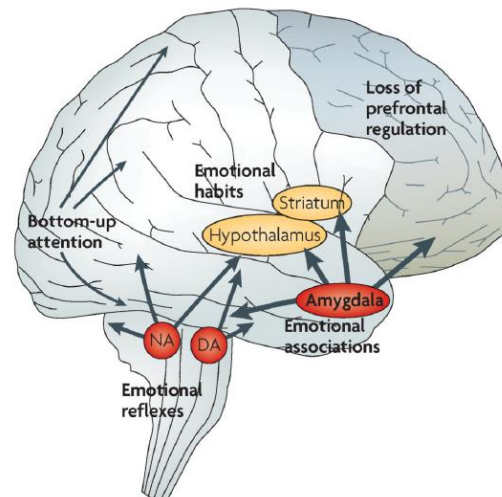

Figure 3. Amygdala control during stress conditions (23)

Some studies with rodents have demonstrated the essential role of the subject's sense of control over the stressor. Subjects who felt in control of the situation (even if this was an illusion) were often not impaired by stress exposure, as the PFC was able to supress the brainstem stress response (34).

The catecholamine actions synergize with glucocorticoid effects in the PFC and in the amygdala. Stress increases the release of glucocorticoids as well as that of catecholamines, and it has been shown that high levels of glucocorticoids by themselves can impair working memory. (32,33,35,36).

Corticotropin-releasing factor (CRF) is an essential mediator of the behavioural, endocrine and autonomic responses to stress. The effects of CRF are mediated by two receptors, corticotropin-releasing factor receptor CRFR1 and CRFR2 that are regulated with mineralocorticoids levels. Strong evidence supports a key role for the dysregulation of CRF pathways in anxiety and depression in humans and animals.

The central nucleus of the amygdala (CeA) is the major extrahypothalamic location of CRF-containing neurons that release CRF in response to stress. The basolateral nucleus of the amygdala (BLA) has been shown to play a key role in emotional arousal and stress-induced, CRF-mediated modulation of cognitive processes. Magnocellular neurons in the BLA express high levels of CRFR1 and project to areas such as the hippocampus, where they contribute to the consolidation of emotional memories. Activation of CRFR1 was shown to increase the excitability of these projection neurons, and thus the release of CRF in the BLA has been postulated to contribute to stress-induced alterations in affective behaviour, including anxiety states linked to chronic stress. (37)

3.7.1. Hypothalamic-pituitary-adrenal axis (HPA)

Stress activates neural circuits that converge on the paraventricular nucleus of the hypothalamus (PVN), this hypothalamic nucleus receives multiple sources of afferent information about external threats and internal physiologic status, interprets them and translates them into outputs that control hormone release, adjust autonomic tone, and influence behaviours that together improve the likelihood of an organism's survival. (38,39)

During initiation of the stress response, Corticotropin-Releasing Factor (CRF, also referred to as Corticotropin-Releasing Hormone or CRH) neurons in the PVN become highly active and release CRF peptide at the median eminence.
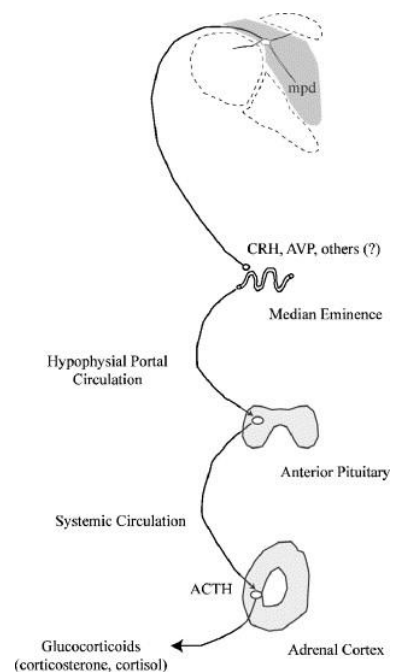

Figure 4. Diagrammatic representations of the HPA axis. (26)

CRH which transits the portal system and signals pituitary corticotropes to release adrenocorticotropic hormone (ACTH) to the circulation (26,40). ACTH, which is the primary effector hormone of the HPA axis, acts on adrenal cortical cells to elevate corticosteroid production, increasing its circulatory concentration. (41)

Elevated cortisol impacts on the function of nearly every cell and tissue in the body by activating glucocorticoid receptors and mineralocorticoid receptors. Excess of cortisol provides negative feedback to the HPA axis, in part, by downregulating CRF and ACTH expression, thereby decreasing HPA axis activity to prevent the deleterious effects of sustained cort signalling and to promote the process of stress recovery(42–44).

3.8. Chronic stress and panic

If glucocorticoids levels are maintained over time, acute stress effects could chronify and produce plastic changes in the brain. Chronic exposure to elevated levels of glucocorticoid hormones has numerous effects on the brain including suppressed neurogenesis and enhanced dendritic pruning in the hippocampus, dendritic shortening in the medial prefrontal cortex, and enhanced dendritic growth in the amygdala, the fear centre of the brain.(45)

In the amygdala, there is expression of high levels of corticotropin-releasing factor receptor CRFR-1 (37). In the pre-frontal cortex, there is also a diminution of brain-derived neurotrophic factor (BDNF), reduction on the expression of MAO-A *(monoamine oxidase A),* a gene which encodes a catabolic enzyme that degrades biogenic amines, including 5-HT, under stress conditions. (46,47)

The changes that occur following stress exposure are dynamic; some of this changes are long-term but only become apparent after an acute stressor is applied (45).

In humans, patients suffering from stress disorders have impaired decision making and behavioural flexibility. For example, high levels of stress are accompanied by difficulty of shifting between strategies in the presence of changes in task demand, and/or are easily distracted by irrelevant stimuli. That inability to change strategies during a task can have detrimental consequences in a person, by affecting personal life and professional performance. Alterations of the connectivity between the brain regions influences by stress might contribute to the etiology of psychopathologies such as generalized anxiety disorder (GAD), social anxiety disorders or post-traumatic stress disorder (PTSD). (25)

It is acknowledged that panic susceptibility is influenced by individual and contextual factors. Ineffective or maladaptive emotion regulation strategies (e.g., worry and rumination) or increased focus on internal processes, lead to reduced attentional resources on skills acquisition and performance. (22)

Chronic stress situations, induces impaired decision making and behavioural flexibility, and when exposed to a non-threatening stimuli there is an exaggerated stress response that becomes maladaptive. (48)

4. JUSTIFICATION

Diving is a popular sport spread worldwide. Although it is a relatively safe sport, fatalities can occur. When this happen, it is important to identify the sequence of events during the diving that led to the fatal outcome.

To identify the causes and circumstances of the death is essential to conduct the investigation with a multidisciplinary team. The coordinate research with the police investigation and the autopsy performed by the forensic pathologist will obtain information that analysed together will be essential to complete the sequence of events that led to the death: triggering factor, disabling agent, disabling injury and finally the cause of death. (13)

In the case of PBt/AGE the investigation will determine the major and minor diagnostic criteria(11). One issue about these criteria is that not all of them have accepted diagnostic methods. Presence of a panic situation, which is one of these criteria, can only be determined by the statement of the people that witnessed the event and sometimes it can be imprecise or limited. (17)

Panic has lately been described by numerous authors as one of the most important triggering factors in diving fatalities, between 40-60% of the cases (9), especially when a rapid ascent is present causing PBt/AGE. Panic affects divers of all ages and both sexes regardless of their experience. Even though it is a significant problem in scuba diving, there has been a tendency for diving organisations to ignore this problem until recent years. Also, editors and authors of introductory scuba books have typically not included discussions of anxiety and panic (17).

It is known that panic is influenced not only for environmental factors, but also by individual factors, especially chronic stress. Chronic stress produces plastic changes especially in the amygdala and pre-frontal cortex. When an unpredicted or a hazardous situation appears there is an extreme stress-response (48) that in the diving situation is maladaptive and can led to a rapid and lethal ascent.

Developing a new rat SCUBA-diving-like model we will determine potential biomarkers (MAOA and CHRH1) useful to elucidate if chronic stress is the underlying cause that lead to wrong decisions like rapid ascent and PBt/AGE fatalities.

To produce chronic stress, we will use chronic unpredictable stress (CUS) protocol as it exposures to a variety of stressors during several weeks that realistically represents the stressors faced by humans in everyday life.

The finding of chronic-stress biological markers will be useful in both legal and social fields. In the legal field, the articles 340, 341, 342 and 343 of *La Ley de Enjuiciamiento Criminal* stablishes that the forensic pathologist must determine the cause of death and its circumstances (49). Determining the molecular parameters of chronic stress, we will be able to stablish the circumstances of death up to the beginning of the sequence of the events.

 In the social branch it could help to identify which subgroups of divers are at elevated risk of injury or death, especially the ones with trait anxiety, and develop preventive interventions in future regulations and guidelines to limit the practice of scuba-diving to high risk groups or give special training to improve behavioural control in stressful situations(8).

This investigation can provide new information about risk factors in the practice of diving that can help to avoid unsafe behaviours and prevent future diving accidents.

5. HYPOTHESIS AND OBJECTIVES

Hypothesis

Chronic stress leads to a maladaptive reactive behaviour during a threatening situation in rat SCUBA-diving-like model. Such wrong decision situation may be associated with alterations in chronic stress-related biomarkers expression in both prefrontal cortex and amygdala

Objectives

- To develop a new rat SCUBA-diving-like model suitable to determine potential biomarkers useful to elucidate underlying cause that lead to wrong decisions like rapid ascent and PBt/AGE fatalities.
- To assess whether chronic stress impairs choice-making behaviour during an underwater threatening situation by means of rat SCUBA-diving test
- To evaluate the correlation between impaired choice-making behaviour and the mRNA expression of stress-related biomarkers MAOA and CHRH1 in the prefrontal cortex and amygdala respectively, by means of qPCR analysis right after the underwater threatening situation.
- To evaluate the suitability of both MAOA and CHRH1 mRNA expression as biomarkers of underwater impaired choice-making behaviour by means of time-course assessment during 12 hours post-mortem interval (0, 6 and 12 hours)

6. MATERIAL AND METHODS

6.1. Animals

A total of 60 Long Evans male rats aged 7 weeks will be purchased from Charles River Laboratories.

They will be kept in the animal facility of the Universitat de Barcelona under standard conditions in a temperature-controlled room (21 ± 2ºC), 60% humidity and 12-hour light/dark cycle with 6:00/18:00 lights on/off.

All the animals will be housed in groups of 2 per cage in Plexiglas cages and supplied with food and water "*ad libitum*" for one week before experiments start.

6.2. Study design and experimental groups
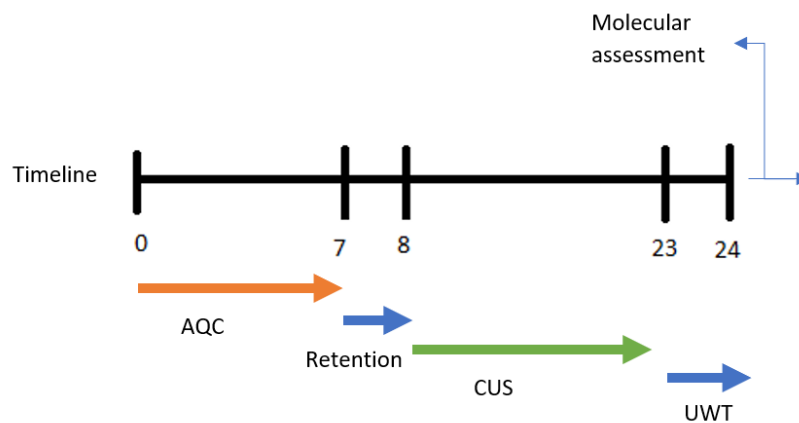
Figure 5 General timeline of the preclinical trial. In black, the days when each phase will conclude. The colour bars represent the different phases of the study. Abbreviations: AQC: acquisition task; CUS: chronic unpredictable stress, UWT: underwater trauma

The design of the study is a pre-clinical animal study.

Rats will firstly be trained to correctly perform a SCUBA-diving test, consisting of an acquisition task (ACQ) followed by a retention (RT) test. During ACQ rats will progressively learn to escape from a water-filled tank using a tube-corridor connected with a dry compartment. RT test will confirm that rats properly learnt the escaping behaviour.

The task will last 7 days, in which each day the tank will contain more water than the day before until it reaches full filling. Each rat will perform at least three trials per day. 24h hours after the las trial (day 8) a retention test will be performed in order to ensure that all rats acquired correctly the escape task.

Afterwards, rats will be divided in two experimental groups: stressed and control. Rats of stressed group will be subjected to chronic unpredictable stress (CUS) protocol for 15 days. Control rats will keep out of stress. Thereafter, all rats will be subjected to SCUBA-diving test under threatening situation (water trauma; UWT) consisting in closing the tube connected with the dry compartment, obligating rats to look for another new escape tube located in the tank.

At the end of experimental period rats will be sacrificed and brains removed to perform molecular assessment.

Additionally, every two days a sample of blood and hair will be taken to state that rats are suffering (or not) stress.

Moreover, experimental groups will be divided in 3 post-mortem times (t=0, 6, 12), consisting of 10 rats each time-point per experimental group.

The experiments will start after one week of handling to get used at human contact avoiding manipulation stress.

6.3. Experimental design

1) First phase: SCUBA-diving AQC test

The rats of both groups will be exposed to the SCUBA-diving AQC task for 7 consecutive days in a plastic tank (diameter 50 cm, height 60cm) that will be connected to another water tank, (75x50 cm), through a tube-corridor at the bottom, where the rat will be able to get out through a ramp. (annex 3)

On the first day, the water tank will be empty, and the rats will be able to find the tunnel and go to the other tank walking. Every day, 4 cm of water (22 ± 2ºC) will be added until it reaches full filling (30 cm deep).

The rats will be exposed to these SCUBA-diving test at least 3 times a day so they can get used to it and can learn to find and use the exit.

Once finished the 7 days, after 24 hours (day 8) a retention test (RT) will be performed in order to ensure that all rats acquired correctly the escape task.

After ACQ the rats won't have contact with water during the 15 days that the CUS is performed. To assure that there is retention of this learning, before starting the trial, we will perform a RT pilot test. A reduced group of rats will perform SCUBA-diving AQC test. After that, one group of them will be exposed after 7 days to the water and the other group after 15

days, to determine if they remember how to use the exit. If the learnt behaviour is extinguished, we can modify the CUS protocol decreasing the duration to 7-day stress protocol and increasing its intensity.

2) Second phase: Chronic unpredictable stress (CUS) (37,50)

For 15 days, rats of the stressed group will be exposed every day to a different stressor that will be applied at different times (between 08:00 and 12:00h). The stressors will be presented randomly to maximize the unpredictability:

- Bright light (300 lx, 30 min)

- Elevated platform (2 h)

- Predator odor (1 h exposure to 10 lL of trimethylthiazoline, a synthetic compound originally isolated from fox feces)

- Smaller cage: Rat pairs will be housed for 4 h in a cage with a 25% reduction of the standard home cage.

- Damp bedding: While rats are temporarily in an empty transfer cage, 200 ml of water will be mixed into 2/3 of the bedding of the home cage. After 6h in the damp bedding, pairs will be transferred to a clean home cage.

- Foreign bedding: Experimental groups will be housed in an empty home cage of other rats. For 12 hours.

This test is used because it has a good validity and it represents the stressors faced by humans in everyday life more realistically.

- **Measurement of corticosterone plasma levels**

Blood samples will be collected via coccigean vein, the day before of the beginning the CUS procedure and every two days during the second phase of the experiment to assess if the rats are suffering (or not) stress.

Total corticosterone in serum samples will be analysed by Cortisol ELISA kit© (DetectX®) according to the manufacturer's instructions.

3) Third phase: Scuba-diving test under threatening situation (UWT)

One day after the CUS is concluded, the two groups of rats will be exposed to the SCUBA-diving test under threatening situation (water trauma; UWT).

The underwater stress experience will be performed into a water tank like the one used for the ACQ, but there will be a new corridor that will connect to a third water tank of the same characteristics of the previously used (75x50 cm). (annex 4)

Rats will start their free swim with the tube of the first tank closed, a special metal net will cover all the surface to avoid that rats get out of the water so rats will be obligated to look for the new escape tube located in the tank.

After 30 seconds, if the rats haven't found the tube the metal net will be released, and the first tube will be opened to let the animal get out of the water.

The experiments will be performed during light hours.

The severity of the procedure is considered of a severe degree because there is an evocation of escape and avoidance reactions where the animal is unable to escape or avoid the stimulus and are expected to result in severe distress. (51)

6.4. Behaviour assessment

The rats will be recorded during the experiment. The camera will be disposed in the roof in the centre of the water tank, and we will compare the behaviour during the UWT between the stressed group and the control group. To assess their behaviour, we will determine:

- Latency to the new open exit. (seconds)
- Number of incorrect attempts to get out.
- Ability to scape (yes/no)

6.5. Tissue excision and processing

After assessing underwater conduct of escape, each experimental group will be euthanised with pentobarbital sodium (dose of 200 mg/kg, and concentration of 40 mg/mL) administered intraperitoneal. (53)

The brain will be removed and semi-frozen by 1-minute covering under dry ice powder. This process will be repeated to remove the brain in different post-mortem interval times (t=0; t=6, t=12h) to determine the stability post- mortem of the markers. During the post-mortem interal the rats will be preserved with an artificial refrigeration process in a refrigerating

Figure 6. *Coronal sections of the rat brain from (56).*

chamber at 4ºC until the moment of their dissection.

In all the cases the isolation of both hippocamps and amygdala will be performed following the atlas of Paxinos and Watson (54) with an sterile 2 mm spatula (52).

The regions that will be dissected are: Pre-frontal cortex (PFC) and amygdala brain regions.

6.6. Molecular assessment

qRT-PCR *(quantitative real time polymerase chain reaction)* will be used to measure all the markers in the amygdala and prefrontal cortex.: MAOA and CRHR1.

 Total RNA will be extracted from brain tissue using an RNeasy Plus Universal Mini Kit (Qiagen, Valencia, CA). Total RNA will be reverse transcribed using TaqMan Reverse Transcription Reagent kit (life technologies corporation, Carlsbad, CA). The primer concentrations will use will be 500nmol and will be obtained from Integrated DNA technologies, Coralville, IA.

7. STATISTICAL ANALYSIS

The statistical analysis will be performed using Statistical Package for Social Sciences (SPSS) and the responsible statistician who will do it will be blinded to the study groups.

We will apply the normality test Kolmogorov-Smirnov (n>30) to determine if there is a normal distribution of the results.

If it is normal we will apply the parametric test T-student to compare molecular expression (MAOA, CHRH1) and the behavioural responses to the underwater trauma between the two groups.

 ANOVA will be used to compare the molecular expression in different situations (stressed, control) and different post-mortem times.

If the distribution is normal we will express the data as mean ± standard error of the mean (SEM).

If the distribution is not normal we will apply the non-parametric ANOVA: Kruskal-Wallis and the U de Mann-Whitney to perform pairs comparisons. In this case, we will express the data as median and interquartile range (IQR).

Differences and correlations will be considered statistically significant when p value < 0.05 defining a confidence interval of 95%.

8. ETHICAL AND LEGAL CONSIDERATIONS

All the experimental procedures and animal husbandry will be conducted following the ARRIVE guidelines and the European Parliament and the Council Directive of 22 September 2010 (2010/63/EU), and will be approved by the Animal Ethics Committee of the University of Barcelona (CEEA-UB).

Its approbation is mediated by the "Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural de la Generalitat de Catalunya".

General state of rats will be assessed with Morton-Griffiths test. (56)

All efforts will be made to minimize the number and suffering of the experimental animals, for this reason the 3'R principle is applied: reduce, refine and replace. The replacement is not possible as the panic reaction we want to analyse is too complex to use another approach without the use of live animals. To achieve the refinement for animals welfare, they will be maintained in proper conditions, as described earlier in the methodological section, and will be taken care by veterinary and qualified carers. Reduction is considered during the design and the number of the rats used.

The study will also have to accomplish the *Real Decreto 1386/2018, de 19 de noviembre*, that modifies the *Real Decreto 53/2013, de 1 de febrero*, where the basic regulations applicable to the animal protection used in experimentation and other scientific purposes including teaching are established in Spain, and the Catalan regulations: *Llei 5/1995 and Decret 164/1998, de 8 de juliol,* where is modificated the *Decret 214/1997* about the use of experimental animal and other scientific purposes.

9. STUDY LIMITATIONS

One limitation may be the lack of retention of the swimming task 15 days after acquisition. Although the bibliography exposed in the present work supports that rats are able to long-lasting consolidate similar acquisition tasks, this limitation can be solved reducing the phase of CUS. That is, to perform a 7-day protocol in which the reduction of time is compensated by increased intensity. A pilot study will be performed using a reduced set of rats to determine retention 15 days after acquisition in order to apply 7-day stress protocol if needed (explained in the **SCUBA-diving AQC test** section).

Another limitation is the results extrapolation of the rat model to human investigation. On one hand, it is expected to use a SCUBA-diving-like model rat that of course SCUBA (diving equipment) is missing. However, considering that the aim of the present work is evaluate whether mood disorders may be the cause that leads to underwater wrong decisions due to panic, we consider this fact not completely relevant. Moreover, it is worth to note that the circumstances of death during the diving can produce some other variables that could confuse the results, for example if the corps is found after 36 hours of death the decomposition artefact would interfere in the molecular determination in the brain. In these cases, our proposed biomarkers would be not suitable, but the hair cortisol determination might be alternative data of indirect analysis of the potential effects of stress on individuals that performed rapid ascent and suffered PBt/AGE fatalities.

Finally, it is important considerate that interindividual differences between rats in the response to stress could exist. That is, some rats could naturally express resilience behaviour and coping stress effects. This limitation will be solved analysing corticosterone in rats plasma during the CUS phase. Only rats presenting increased corticosterone in blood at the end of CUS phase will be included in the Stressed experimental group.

10. WORK PLAN

The research team will develop the tasks of coordination, execution of the experiment, interpretation and presentation of the results.

This investigation will be conducted by the Research Group on Clinical Anatomy, Embryology and Neuroscience (NEOMA). The principal investigators of this group are:

Josep M. Casadesús; Pere Boadas-Vaello; Anna Carrera; Maite Serrando; Fernando Aguirre; Núria Carbó; Francisco Reina

The tasks will be developed at the laboratory of the Clinical Anatomy, Embryology and Neuroscience (**NEOMA**) research group of the Medical Sciences department of the University of Girona (UdG), located in the Girona Science and Technology Park and at the animal facilities of the Universitat de Barcelona (UB).

The project writing and the ethical committee evaluation was performed before starting the preclinical trial.

The sequence of activities will be developed in the following order:

- o **Stage 1. Animal experimentation and molecular assessment.**

January - March (2020)

The animal experiments will start during January in the UB laboratories, after the week of accommodation in the animal facility.

The experiment will be performed in the following order:
- Acquisition task (AQC): 7 days
- Retention: 1 day
- Chronic umpredictable stress (CUS) protocol: 15 days to the stressed group, the non-stressed will remain in its cage unaltered.
- SCUBA-diving test under threatening situation (UWT): 1 day. The experiment will be performed with the behavioural assessment.

After the experiment, the qPCR will be performed to assess the presence or absence of CHRH1 and MAOA.

This experiment will last 3 months and the proteins will be determined in a post-mortem time of t=0.

The total duration of the experiment will be of 6 months, as we will have to repeat the same procedure in each post-mortem time. (t= 6, t=12)

The principal investigators will conduct the experiment.

- o **Stage 2. Statistical analysis and interpretation of the results**

October (2020)

The statistical analysis will be performed by an experienced statistic. All the information collected will be analysed according to the variables of our study.

The results will be assessed by the principal investigators.

- o **Stage 3. Final report elaboration**

November (2020)

After the interpretation of the results the conclusion and discussion of the experiment will be written, and the final report will be elaborated by the principal investigators.

- o **Stage 4. Publication of the results**

December (2020)

The principal investigators will write a paper to publish the results and will disseminate the study by a conference and publication to forensic journals.

CHRONOGRAM

	STAGE				2020									
		Jan	Feb	March	April	May	June	July	August	Sept	Oct	Nov	Dec
1	Animal experimentation, qPCR and hair cortisol determination	t=0											
					t=6								
								t=12					
2	Statistical analysis and interpretation of results												
3	Final report elaboration												
4	Publication and difussion of the results												

11. BUDGET

Item	Subtotal
Fungible	
Purchase of the rats (60 rats x 38.89€ u)	2.334€
qPCR material and microRNAs	6. 500€
Cortisol determination kit	750€
Dry ice (5 Kg (70€) x 3 months)	210€
Purchase freezing medium to cut tissue samples in cryostat (Tissue Freezing Medium)	400€
Standard laboratory reagents and other equipment	3.000€
CUS experiment	1000€
Water tank	1500€
Video camera and memory cards	700€
Others	
Maintenance of animals (5,41€ cage x week)	3.895€
Standard laboratory reagents and other equipment (i.e. warm lamp)	2.250€
Staff expenses	
Statistic (30h x 35 €/h)	1050€
Publication and dissemination	
Revision and publication fees	1000 €
Dissemination (assistance to congress)	860€
	18.949,21 €
Cànon Udg	+21%
	22.928,29€

12. FEASIBILITY

The research team that will participate in the present project is a multidisciplinary group of researchers consisting of preclinical researchers and specialists in Forensic Pathology, all of them with wide expertise in their fields providing the necessary knowledge towards objectives achieving. It is worthy to note also, that preclinical researchers have the official accreditation of the Generalitat de Catalunya that allows them to work with experimental animals, an indispensable requirement to carry out the project.

Furthermore, *in vivo* experiments will be performed in the Animal Facilities of the Universitat de Barcelona, paying public university fees that cheapen the global project costs. On the other hand, all other scheduled molecular experiments, will be conducted in the laboratory of the Clinical Anatomy, Embryology and Neuroscience (**NEOMA**) research group of the Medical Sciences department of the University of Girona (UdG), located in the Girona Science and Technology Park, which is fully equipped to perform such tasks. Both facilities may ensure successful assessments of the present study tasks.

Moreover, the present work is supported by the IMLCFC Forensic Pathology Service, the Center of Forensic Pathology of Girona, and such collaboration will be really useful to properly extrapolate results and/or further study the potential applicability to human samples.

Finally, but not least, the budget is sustainable for a one-year project and the project fits into the scopes of several research foundations as well as public Spanish Government scientific calls. That is, both objectives and budget meet public and private scientific calls requirements to apply for supporting, and this fact may suggest that the project budget could be successfully funded in a near future.

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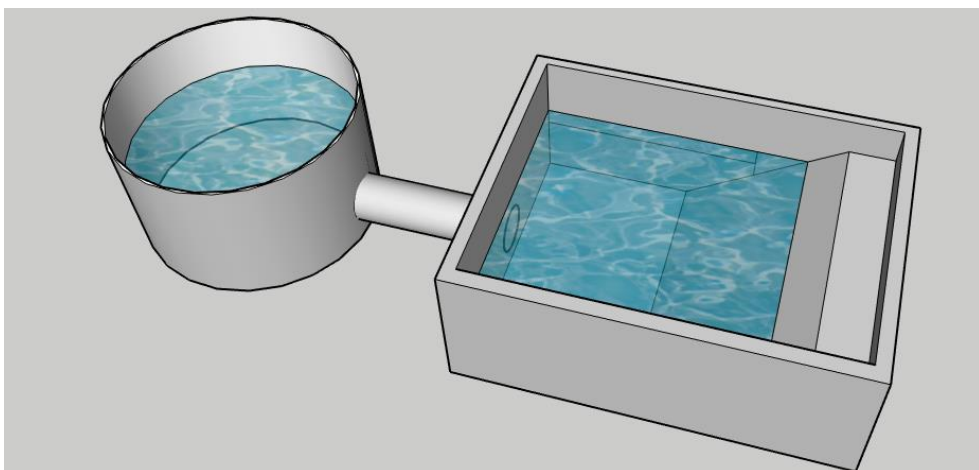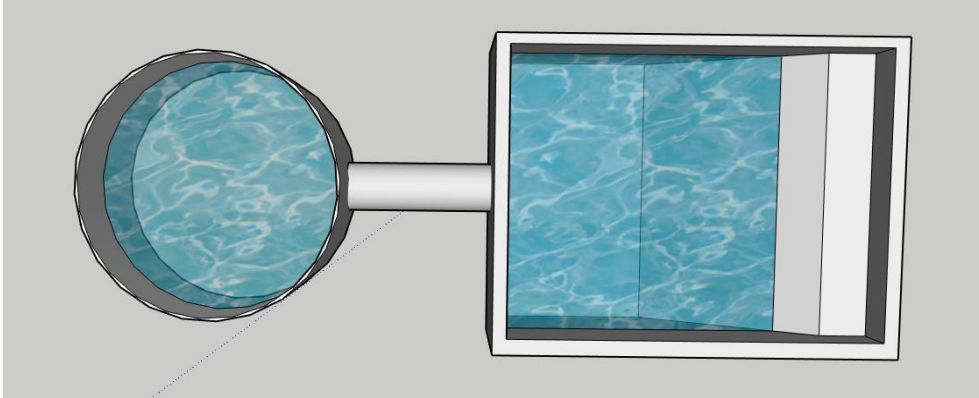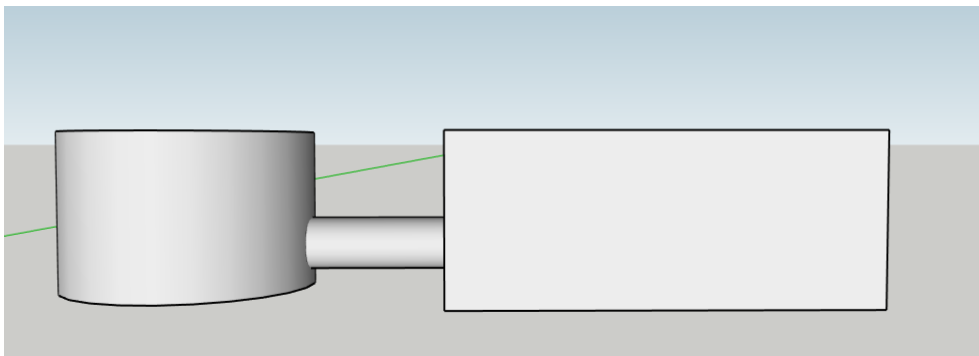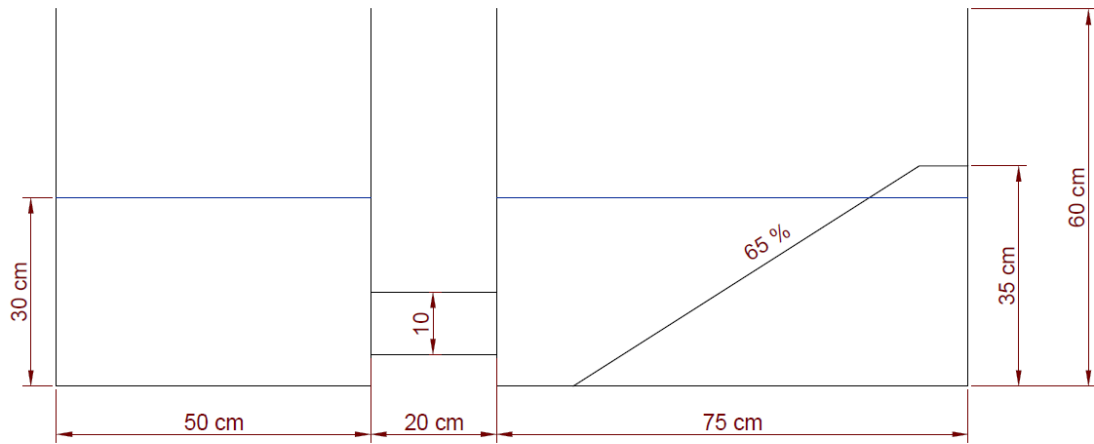
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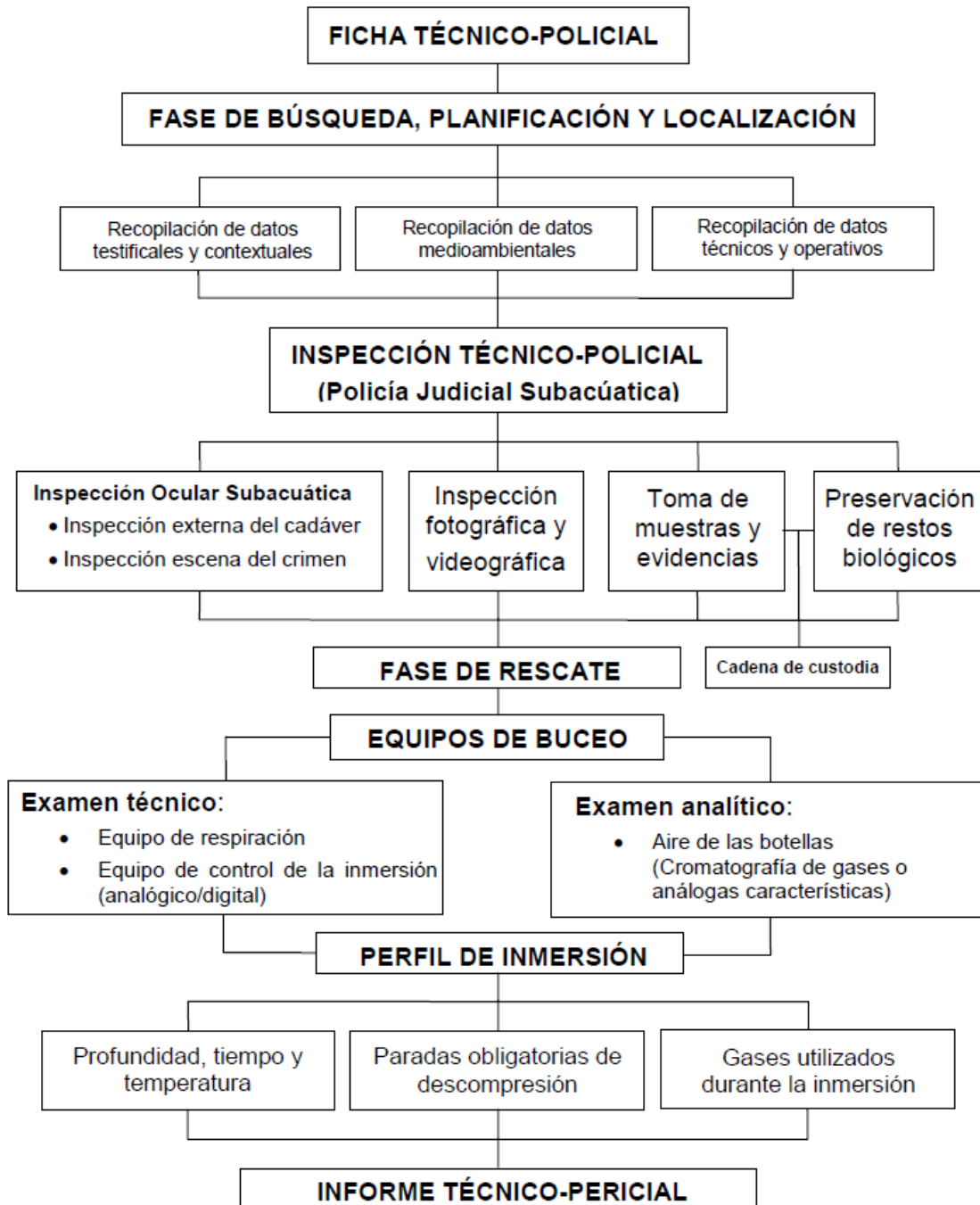
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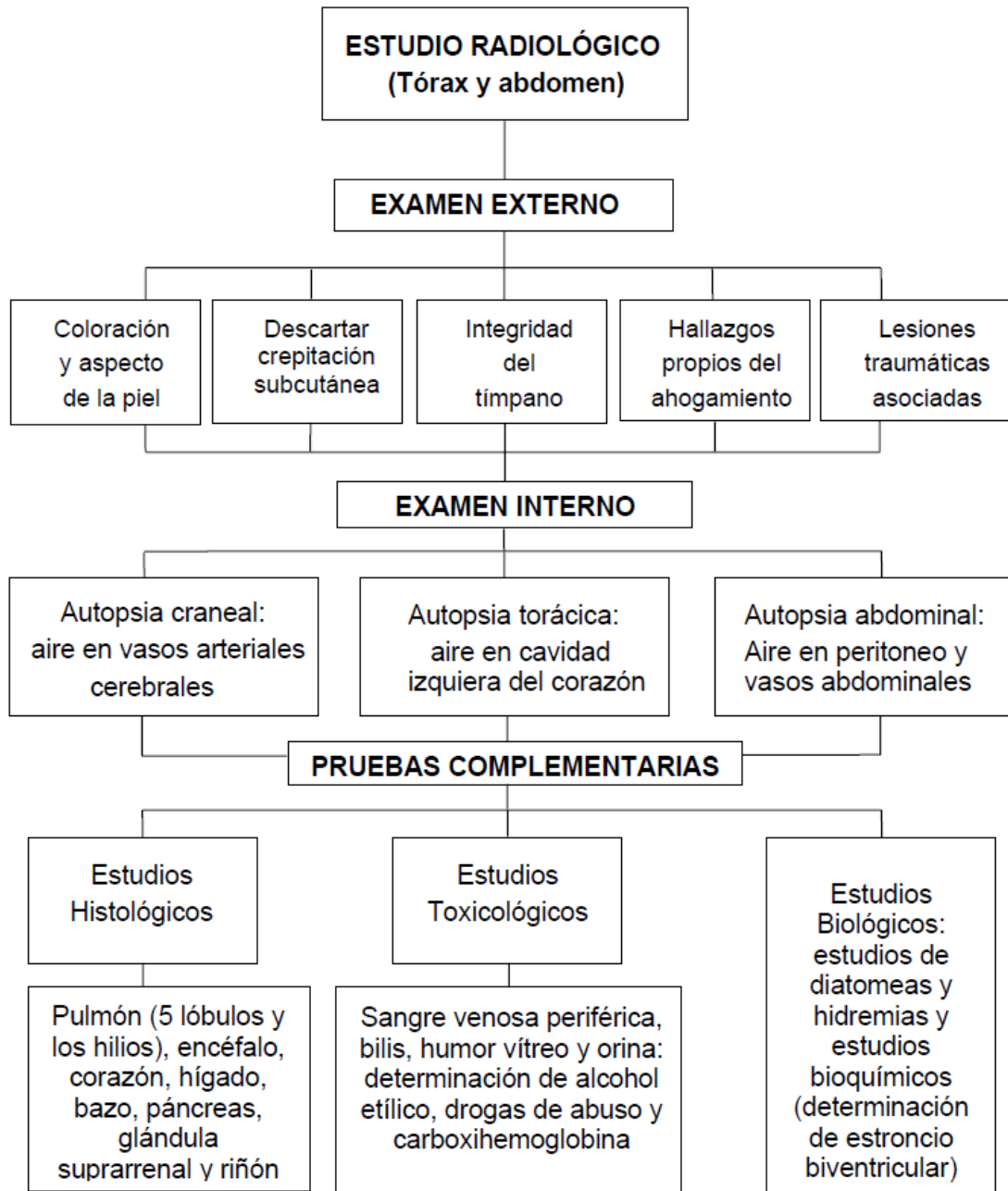
Annex 2. *Autopsy protocol in suspect cases of PBt/AGE in SCUBA-diving deaths. Adapted from (7)*

Annex 3. Water tank design for the AQC (Author: Maria Carbó)

Annex 4. Water tank design for the SCUBA-diving test under threatening situation (UWT). (Author: Maria Carbó)