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# EFFECTS OF EARLY DEXAMETHASONE TREATMENT ON SEVERAL MARKERS OF INFLAMMATION AND FIBROSIS IN AN ANIMAL MODEL OF LUNG SILICOSIS IN RATS – A PILOT STUDY

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## Abstract

Lung silicosis is primarily caused by inhalation of particles of silicon oxide (silica). Despite a huge progress in understanding the interactions among the pathomechanisms of lung silicosis in the last years, there is a lack of effective therapy. With respect to a wide therapeutic action of corticosteroids, the purpose of this pilot study was to evaluate early effects of dexamethasone on several markers of inflammation and lung fibrosis in a rat model of silicosis. The silicosis model was induced by a single transoral intratracheal instillation of silica (50 mg/ml/animal), while the controls received an equivalent volume of sterile saline. The treatment with intraperitoneal dexamethasone initiated the next day after the silica instillation and was given 2-times a week at a dose of 1 mg/kg, while the controls received an equivalent volume of saline. The animals were euthanized 14 or 28 days after the treatment onset. Total and differential counts of leukocytes in the blood and bronchoalveolar lavage (BAL) fluid were determined. The presence of collagen in the bronchioles and lung vessels was detected by Sirius red staining and a smooth muscle mass was detected by smooth muscle actin. In comparison to saline, the instillation of silica increased the total count of circulating leukocytes after 14 and 28 days of the experiment (both $p<0.05$), which was associated with higher counts of lymphocytes ($p<0.05$ after 14 days, $p>0.05$ after 28 days) and slight but non-significant increases in neutrophils and eosinophils (both $p>0.05$). Although the total cell count in the BAL fluid did not change significantly, the percentages and absolute counts of neutrophils, eosinophils, and lymphocytes ($p<0.05$, $p<0.01$ or $p<0.001$) elevated after 14 and 28 days of the experiment. Silica induced an accumulation of collagen in the bronchioles ($p<0.001$ after both 14 and 28 days) and pulmonary vessels ($p<0.01$ after both 14 and 28 days) and elevated a formation of smooth muscle mass ($p<0.05$ after 14 days, $p<0.01$ or $p<0.001$ after 28 days). Treatment with dexamethasone decreased circulating leukocytes ($p<0.01$) and lymphocytes ($p<0.001$) and increased neutrophils ($p<0.05$), which was associated with a slightly decreased total cell count in BAL ($p>0.05$), decline in lymphocytes ($p<0.01$), and slight decreases in neutrophils and eosinophils after 28 days of the treatment. Moreover, dexamethasone reduced the accumulation of collagen ($p<0.01$ after 14 days and $p<0.001$ after 28 days) and the formation of smooth muscle mass ($p<0.01$ for bronchioles and $p>0.05$ for vessels after 24 days, $p<0.001$ for both bronchioles and vessels after 28 days). In conclusion, early dexamethasone treatment mitigated silica-induced granulocytic-lymphocytic inflammation and decreased a formation of collagen and smooth muscle mass in the bronchiolar and vascular walls, demonstrating a therapeutic potential of dexamethasone in the lung silicosis.

**Key words:** lung silicosis, animal model, inflammation, oxidative stress, dexamethasone

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## INTRODUCTION

Lung silicosis, a serious fibrotizing disease, is primarily caused by massive or long-term inhalation of particles of silicon oxide (or silica) (1). Background of the pathological changes in silica-injured lungs has not been completely understood; however, they presumably result from direct cytotoxic effect of silica on macrophages, activation of macrophage surface receptors, lysosomal rupture, generation of reactive oxygen species (ROS), activation of inflammasome, overproduction of cytokines and chemokines, cell apoptosis/pyroptosis, and ongoing lung fibrosis (2-5). Nevertheless, despite a huge progress in understanding the complex interactions among the proinflammatory, prooxidant and profibrotic mechanisms in the pathogenesis of lung silicosis in the last years, there is still a lack of effective therapy. Various anti-inflammatory, antioxidant, and anti-fibrotic treatments including herbal compounds have been tested in experimental models of lung silicosis as well as in patients suffering from lung silicosis; however, effects of the given treatments were often contradictory (6-8).

With respect to a wide therapeutic action of a long-acting corticosteroid dexamethasone, the purpose of this pilot study was to evaluate early effects of dexamethasone on several markers of inflammation and lung fibrosis in a rat model of silicosis. We have presumed that early administration of dexamethasone may positively influence a development of the inflammation and thereby could mitigate the inflammation-associated lung fibrosis.

## METHODS

This study was approved by the National Veterinary Board of Slovakia (Ro-1068/19-221/3) and the local Ethical Committee of Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava (EK 6/2019). Adult male Wistar rats of the mean body weight of 270-320 g were supplied by a certified animal breeding station (VELAZ, Czech Republic). Animals were kept in a certified faculty animal house where they underwent a 7-day quarantine and acclimation, with available food and water *ad libitum*.

Then, the animals (n=48 in total) were divided into three groups. In Sil group of animals, a model of lung silicosis was induced by a single transoral intratracheal instillation of silica (50 mg/ml/animal) in the inhalation anesthesia (4% isoflurane), while the control animals (Sal group) received an equivalent volume of sterile saline. The instillation of silica/saline was performed on an angled board by hooking the front teeth while positioning of the tongue laterally enables the instillation of silica suspension/saline by micropippetor into the trachea through opened vocal cords during inspiration (modified according to 9,10). In a half of the silica-instilled animals, the treatment with intraperitoneal dexamethasone (Dexamed, Medochemie, Cyprus) initiated the next day after the silica instillation and was given twice a week at a dose of 1 mg/kg (Dex group). The animals were euthanized 14 days (Sil14, Sal14, and Dex14 subgroups, each of n=8) or 28 days (Sil28, Sal28, and Dex28 subgroups, each of n=8) after the treatment onset by an overdosing of anesthetics (Zoletil, Virbac, France). The analysis after 14 days enables an observation of inflammatory changes, while the analysis after 28 days reveals an observation of early fibrotic changes (11,12).

Sample of blood was taken by a direct puncture of the heart. The total count of leukocytes was determined by a veterinary hematologic analyzer (Sysmex XT-2000iV, Japan). The differential count of leukocytes was estimated microscopically after staining by May-Grünwald/Giemsa-Romanowski and expressed in percents (%) and in absolute values (x $10^9$/L).

The lung was excised. The left lung was lavaged with saline (0.9% NaCl, 2x10 ml/kg b.w.). The total count of cells was measured by a cell analyzer Countess (Thermo Fisher Scientific, USA). Then, the BAL fluid was centrifuged at 1500 rpm for 15 min. The differential

count of cells in the BAL fluid sediment was evaluated microscopically after staining by May-Grünwald/Giemsa-Romanowski and expressed in percents (%) and in absolute values (x $10^3$/mL).

The right lung was washed by saline and stored in 4% formaldehyde. The presence of collagen (by Sirius red staining) and smooth muscle mass (detection of smooth muscle actin, SMA) in the walls of bronchioles and vessels of the right lung was verified immunohistochemically by a qualified histologist. The lung tissue was dehydrated through the series of graded ethanol baths, infiltrated with paraffin and cut into 4µm thick sections.

Detection of collagen by Sirius red: after deparaffinization the slides were firstly stained with Weigert's hematoxylin for visualization of the cell nuclei. After washing in running tap water (10 min), the slides were stained in picro-sirius red solution (Millipore Sigma, USA) for 1 hour. After washing twice in acidic distilled water, and dehydration in 100% ethanol, the slides were mounted in Entellan (Millipore Sigma, USA). The result of staining was red collagen fibers on pale yellow background. The slides were viewed with an Olympus BX43 microscope (Olympus, Japan). The image capture and airway wall thickness measurement was performed with the Quick Photo Micro software, version 3.2 (Olympus, Japan).

Detection of smooth muscle mass by SMA: after deparaffinization, revitalisation and rehydratation, the tissue slides were treated with 3% $H_2O_2$ solution for 10 min for blocking endogenous peroxidases. Washing with Tris buffer was used after each handling step. The sections were incubated with the primary rabbit polyclonal smooth muscle actin (SMA; 1:300, Cell Signaling Technology, USA) for 30 min at room temperature. The slides were then incubated by sequential 10 min incubation with LSAB2 System-HRP for use on rat specimens (Dako, Denmark), which detects primary mouse and rabbit antibodies. The sections were then counterstained with Mayer´s hematoxylin (Himedia Laboratories, India) and mounted with an Entellan (Merck, USA). The sections were viewed with an Olympus BX43 microscope (Olympus, Japan) equipped with a photo camera Canon E0S 2000D. The Quick Photo Micro program, version 3.2 (Olympus, Japan) was used to image capture the sections and measure the thickness of the smooth muscle layer in the bronchial wall and the *tunica media* in the blood vessel wall (expressed by dark brown cytoplasm of SMA-positive cells).

For analysis of the data, the statistical package SYSTAT for Windows (Systat Software Inc., USA) was used. Differences among the groups were analyzed by one-way ANOVA with posthoc Fisher's LSD test. A value of $p<0.05$ was considered statistically significant. The data are expressed as means ± SD.

## RESULTS

### *Total count of leukocytes in the blood*

Determination by a cell counter revealed that the total count of leukocytes did not change in the saline-instilled animals (3.68±1.06 x $10^9$/L in Sal28 vs 3.65±1.24 x $10^9$/L in Sal14, $p>0.05$). Contrary, significant increases were found for the silica-instilled vs the saline-instilled animals after 14 days (5.37±1.19 x $10^9$/L in Sil14 vs 3.65±1.24 x $10^9$/L in Sal14, $p<0.05$) as well as after 28 days of the experiment (4.96±1.18 x $10^9$/L in Sil28 vs 3.68±1.06 x $10^9$/L in Sal28 $p<0.05$). The administration of dexamethasone treatment caused a decrease in circulating leukocytes after 14 days (3.22±1.31 x $10^9$/L in Dex14 vs 5.37±1.19 x $10^9$/L in Sil14, $p<0.01$) as well as after 28 days of the experiment in comparison to the non-treated silica-instilled animals (2.98±1.31 x $10^9$/L in Dex28 vs 4.96±1.18 x $10^9$/L in Sil28, $p<0.01$).

### *Differential count of leukocytes in the blood*

Analysis of the differential count of leukocytes in the blood did show any significant differences between the Sal28 vs Sal14 groups (all $p>0.05$) neither in percentual nor in absolute expressions (Fig. 1, Table 1). Contrary, the instillation of silica resulted in a significant

decrease in a percentage ($p$<0.05) but non-significant decrease expressed in absolute numbers ($p$>0.05) of circulating monocytes in Sil14 vs Sal14 group (Fig. 1, Table 1). In addition, silica instillation after 14 days caused increases in absolute numbers of blood lymphocytes ($p$<0.05), neutrophils ($p$=0.056), and eosinophils ($p$>0.05) compared to the saline-instilled animals (Table 1). After 28 days, relative ($p$<0.05, Fig. 1) and absolute ($p$>0.05, Table 1) values of monocytes in the blood elevated in Sil28 vs Sil14 group. Similar but non-significant trend was also observed for percentages of eosinophils and lymphocytes while neutrophils had a slight tendency to decrease (all $p$>0.05, Fig. 1). These changes were consistent with changes in the absolute values of eosinophils and neutrophils, but the absolute counts of lymphocytes slightly decreased (all $p$>0.05, Table 1).

The administration of dexamethasone after 14 days resulted in increased percentages of circulating neutrophils and monocytes and decreased lymphocytes in comparison to the non-treated animals (Dex14 vs Sil14, all $p$<0.05, Fig. 1). This trend is consistent with increases in absolute numbers of neutrophils, monocytes, and eosinophils (all $p$>0.05), while the number of circulating lymphocytes declined ($p$<0.001, Table 1). After 28 days of the treatment delivery, the elevated percentages of circulating neutrophils and decreased lymphocytes (both $p$<0.05) were found in dexamethasone-treated compared to thenon-treated animals (Dex28 vs Sil28, Fig. 1). The analysis of absolute numbers confirmed a decrease in lymphocytes ($p$<0.001) and an increase in neutrophils ($p$<0.05) as well as slightly decreased counts of monocytes and eosinophils (both $p$>0.05) in Dex28 vs Sil28 groups (Table 1).

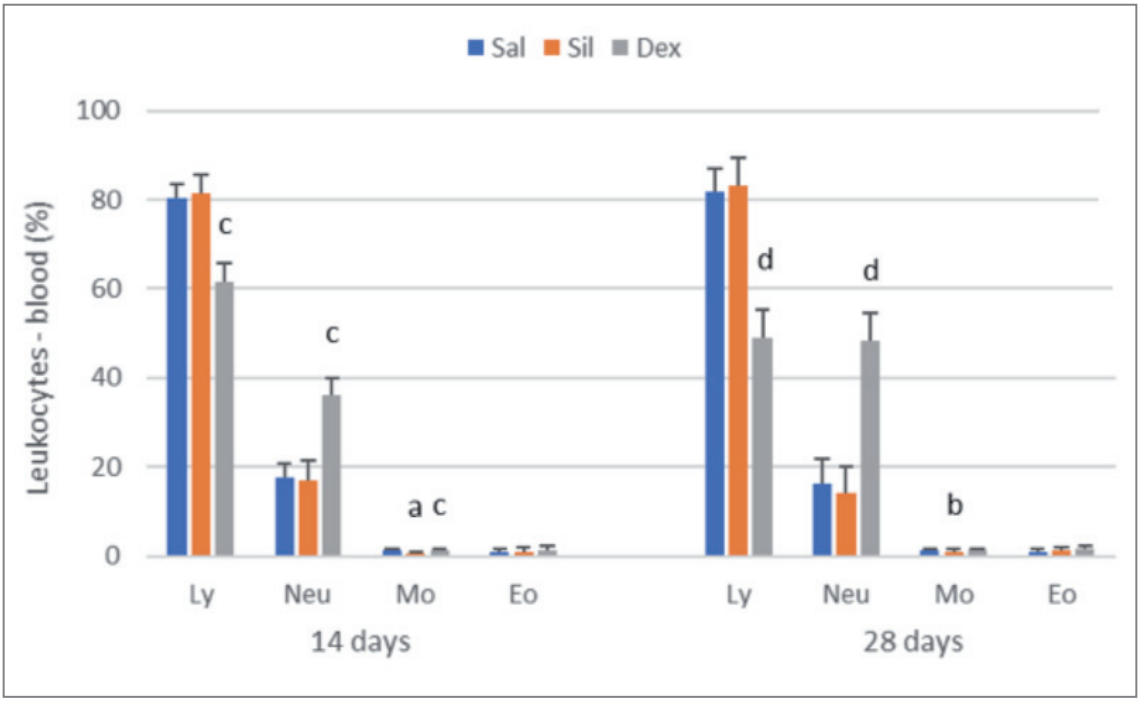


**Fig. 1** Differential count of leukocytes in the blood (%) in the control groups with saline instead of silica (Sal), in the silica-injected and non-treated animals (Sil), and in the silica-injected and dexamethasone-treated animals (Dex) after 14 or 28 days of the treatment delivery.

Abbreviations: Ly: lymphocytes, Neu: neutrophils, Mo: monocytes, Eo: eosinophils. Statistical differences among the groups: [a] $p$<0.05 for Sil14 vs Sal14; [b] $p$<0.05 for Sil28 vs Sil14; [c] $p$<0.05 for Dex14 vs Sil14; [d] $p$<0.05 for Dex28 vs Sil28.

**Table 1** Absolute counts of leukocytes in the blood and in the BAL fluid in the control groups with saline instead of silica (Sal), in the silica-injected and non-treated animals (Sil), and in the silica-injected and dexamethasone-treated animals (Dex) after 14 or 28 days of the treatment delivery.

| | 14 days | | | 28 days | | |
|---|---|---|---|---|---|---|
| **Leukocytes in the blood** (x $10^9$/L) | | | | | | |
| | **Sal14** | **Sil14** | **Dex14** | **Sal28** | **Sil28** | **Dex28** |
| Ly | 2.95±1.07 | 4.33±0.84 [a] | 1.99±0.87 [g] | 3.21±0.83 | 4.18±1.23 | 1.42±0.53 [j] |
| Neu | 0.63±0.19 | 0.97±0.40 | 1.14±0.40 | 0.61±0.22 | 0.67±0.22 | 1.49±0.79 [h] |
| Mo | 0.04±0.02 | 0.03±0.01 | 0.04±0.02 | 0.05±0.03 | 0.05±0.02 | 0.03±0.01 |
| Eo | 0.03±0.03 | 0.04±0.04 | 0.05±0.05 | 0.03±0.04 | 0.06±0.04 | 0.04±0.02 |
| **Leukocytes in the BAL fluid** (x $10^3$/mL) | | | | | | |
| | **Sal14** | **Sil14** | **Dex14** | **Sal28** | **Sil28** | **Dex28** |
| Ma | 97.7±41.5 | 95.4±37.7 | 146±38.8 [f] | 107±45.9 | 102±43.8 | 84.3±42.4 |
| Neu | 2.29±1.66 | 14.3±3.83 [c] | 35.1±49.9 | 3.51±1.35 | 26.8±24.1 [d] | 8.68±5.23 |
| Eo | 0.25±0.32 | 0.81±0.23 [b] | 1.47±1.57 | 0.23±0.23 | 2.39±2.17 [d] | 0.62±0.35 [h] |
| Ly | 0.99±0.64 | 2.35±1.70 | 4.06±2.10 | 0.71±0.27 | 4.92±2.89 [e] | 1.40±0.87 i |

Abbreviations: Ly: lymphocytes, Neu: neutrophils, Mo: monocytes, Eo: eosinophils, Ma: macrophages. Statistical differences among the groups: [a] $p<0.05$, [b] $p<0.01$ and [c] $p<0.001$ for Sil14 vs Sal14; [d] $p<0.05$ and [e] $p<0.01$ for Sil28 vs Sal28; [f] $p<0.05$ and [g] $p<0.001$ for Dex14 vs Sil14; [h] $p<0.05$, [i] $p<0.01$ and [j] $p<0.001$ for Dex28 vs Sil28.

### *Total count of cells in the BAL fluid*

No significant differences in total counts of cells in the BAL fluid in the saline-instilled animals after 28 days vs 14 days were found (111.7±47.1 x $10^3$/mL in Sal28 vs 101.3±43.6 x $10^3$/mL in Sal14, $p>0.05$). Similarly, no significant differences were detected for comparison of the silica-instilled vs the saline-instilled animals after 14 days (112.9±35.5 x $10^3$/mL in Sil14 vs 101.3±43.6 x $10^3$/mL in Sal14, $p>0.05$) or after 28 days of the experiment (135.7±68.5 x $10^3$/mL in Sil28 vs 111.7±47.1 x $10^3$/mL in Sal28 $p>0.05$). Dexamethasone treatment showed a trend to increase count of BAL cells after 14 days (186.3±43.6 x $10^3$/mL in Dex14 vs 112.9±35.5 x $10^3$/mL in Sil14, $p<0.05$) but non-significantly decreased the total cell count after 28 days of the experiment in comparison to the non-treated silica-instilled animals (95.0±43.8 x $10^3$/mL in Dex14 vs 135.7±68.5 x $10^3$/mL in Sil28, $p>0.05$).

### *Differential count of leukocytes in the BAL fluid*

Differential count of leukocytes in the BAL fluid did not change significantly between the Sal14 vs Sal28 groups (all $p>0.05$, Fig. 2 and Table 1). Contrary, in response to the instillation of silica an increase in lymphocyte percentage ($p<0.05$) as well as a slight elevation in an absolute count ($p=0.065$) of lymphocytes was observed in Sil28 vs Sil14 group (Fig. 2, Table 1).

More obvious changes were determined for the silica-instilled animals vs the saline-instilled controls after 14 days of the treatment delivery (Sil14 vs Sal14) as expressed by a decrease in

percentage of macrophages ($p<0.001$), while the absolute numbers of macrophages remained unchanged ($p>0.05$). In addition, silica instillation caused increases in percentages and absolute numbers of neutrophils (both $p<0.001$), increases in percentages ($p<0.001$) and absolute numbers ($p<0.01$) of eosinophils, and elevation in percentage ($p<0.05$) and absolute counts ($p=0.054$) of lymphocytes in Sil14 vs Sal14 groups (Fig. 2, Table 1).

Similar differences were also observed after 28 days of the treatment delivery where the percentage of macrophages was lower ($p<0.001$), while the absolute numbers of macrophages remained unchanged ($p>0.05$). However, increases in relative and absolute counts of other cells persisted till the end of the experiment as demonstrated on increases in the percentage ($p<0.001$) and absolute numbers ($p<0.05$) of neutrophils, percentage and absolute numbers of eosinophils (both $p<0.05$), and percentage ($p<0.001$) and absolute numbers ($p<0.01$) of lymphocytes in Sil28 vs Sal28 group (Fig. 2, Table 1).

The treatment with dexamethasone had no effect on percentages of BAL cells after 14 days (for Dex14 vs Sil14 all $p>0.05$), but revealed slight increases in absolute counts of macrophages ($p<0.05$), neutrophils, eosinophils, and lymphocytes (all $p>0.05$). However, the situation dramatically changed after 28 days of the treatment delivery when a percentage of macrophages increased ($p<0.05$) but the absolute number of macrophages slightly decreased ($p>0.05$). In addition, in the comparison of Dex28 vs Sil28 groups we found a decrease in percentage and absolute counts of lymphocytes (both $p<0.01$), a slight but non-significant decreases in percentages of neutrophils ($p=0.061$) and eosinophils ($p=0.057$), which was associated with decreases in their absolute values ($p<0.05$ for eosinophils and $p=0.056$ for neutrophils) (Fig. 2, Table 1).

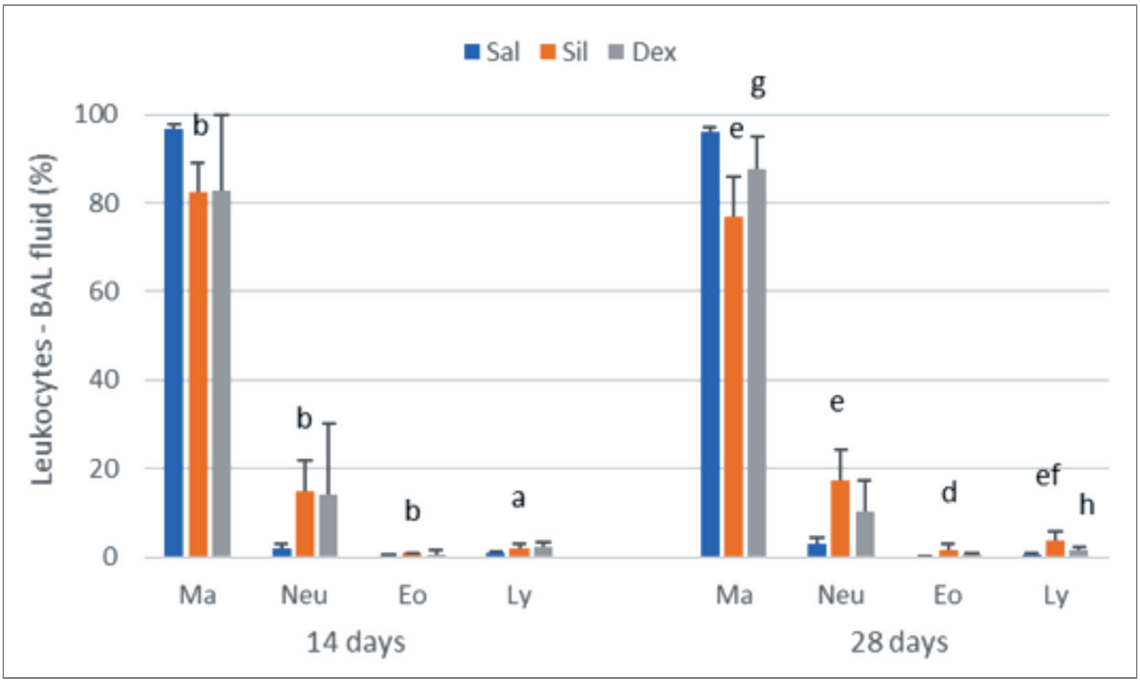


**Fig. 2.** Differential count of leukocytes in the BAL fluid (%) in the control groups with saline instead of silica (Sal), in the silica-injected and non-treated animals (Sil), and in the silica-injected and dexamethasone-treated animals (Dex) after 14 or 28 days of the the treatment delivery.

Abbreviations: Ma: macrophages, Neu: neutrophils, Eo: eosinophils, Ly: lymphocytes. Statistical differences among the groups: [a] $p<0.05$ and [b] $p<0.001$ for Sil14 vs Sal14; [d] $p<0.05$ and [e] $p<0.001$ for Sil28 vs Sal28; [f] $p<0.05$ for Sil28 vs Sil14; [g] $p<0.05$ and [h] $p<0.01$ for Dex28 vs Sil28.

*Collagen accumulation in the bronchioles and pulmonary vessels*

Immunohistochemical analysis by Sirius red staining showed a slightly increased accumulation of collagen in bronchioles and pulmonary vessels in Sal28 vs Sal14 groups (both $p<0.05$). Similar trend of higher collagen accumulation was detected in Sil28 vs Sil14 groups; however, the differences were not statistically significant (both $p>0.05$) (Fig. 3). The production of collagen obviously raised in the silica-instilled animals in comparison to the controls (Sil vs Sal) where the increase was statistically significant in bronchioles ($p<0.001$) and vessels ($p<0.01$) after both 14 days and 28 days of the treatment delivery. The treatment with dexamethasone reduced formation of collagen compared to the non-treated animals already after 14 days of the experiment ($p<0.01$ for both bronchioles and vessels, Dex14 vs Sil14) and this trend was even more obvious after 28 days of the treatment delivery ($p<0.001$ for both bronchioles and vessels, Dex28 vs Sil28) (Fig. 3).

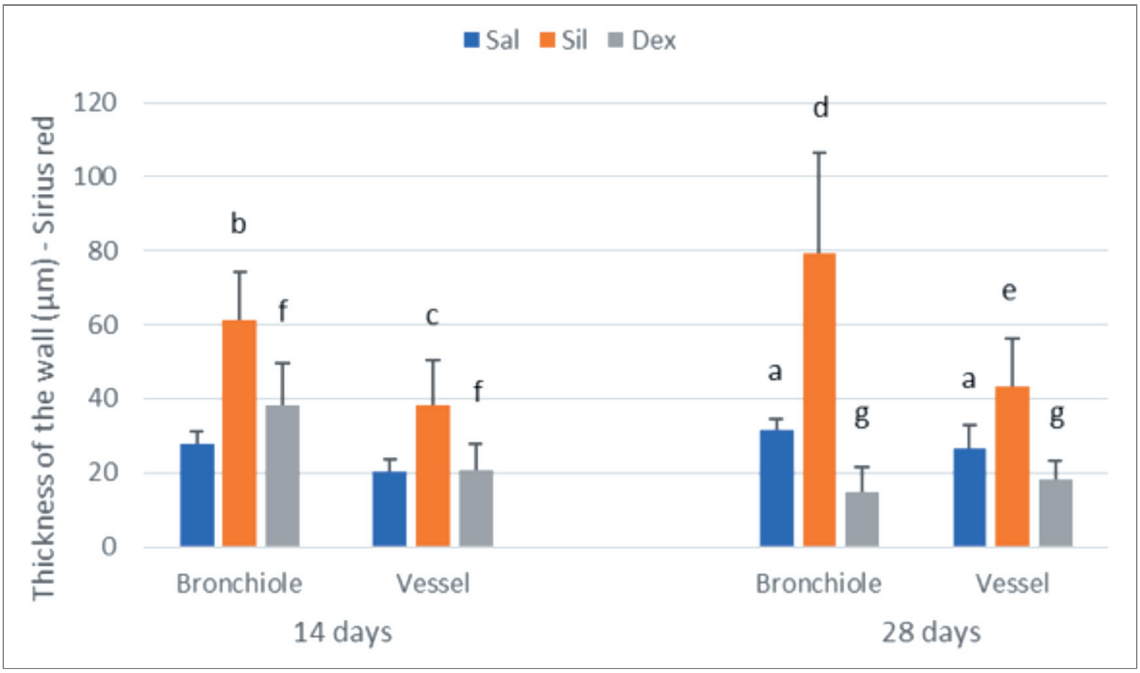


**Fig. 3.** Thickness of the wall of bronchioles and pulmonary vessels (in µm) – staining for presence of collagen by Sirius red in the control groups with saline instead of silica (Sal), in the silica-injected and non-treated animals (Sil), and in the silica-injected and dexamethasone-treated animals (Dex) after 14 or 28 days of the treatment delivery.

Statistical differences among the groups: [a] $p<0.05$ for Sal28 vs Sal14, [b] $p<0.001$ for Sil14 vs Sal14, [c] $p<0.01$ for Sil14 vs Sal14, [d] $p<0.001$ for Sil28 vs Sal28, [e] $p<0.01$ for Sil28 vs Sal28, [f] $p<0.01$ for Dex14 vs Sil14, [g] $p<0.001$ for Dex28 vs Sil28.

*Smooth muscle mass accumulation in the bronchioles and pulmonary vessels*

Immunohistochemical analysis by means of antibody against SMA showed no significant differences for Sal28 vs Sal14 or Sil28 vs Sil14 (all $p>0.05$). Silica instillation caused an increase in the smooth muscle mass in the wall of bronchioles and pulmonary vessels compared to the controls, which was statistically significant already after 14 days ($p<0.05$ for both bronchioles and vessels, Sil14 vs Sal14), and this trend aggravated after 28 days of the treatment delivery ($p<0.01$ for bronchioles and $p<0.001$ for vessels, Sil28 vs Sal28) (Fig. 4). The dexamethasone treatment prevented the increase in smooth muscle mass in

bronchioles and vessels after 14 days of the treatment delivery ($p<0.01$ for bronchioles and $p>0.05$ for vessels, Dex14 vs Sil14), and the effect was even more obvious after 28 days of the treatment delivery ($p<0.001$ for both bronchioles and vessels, Dex28 vs Sil28) (Fig. 4).

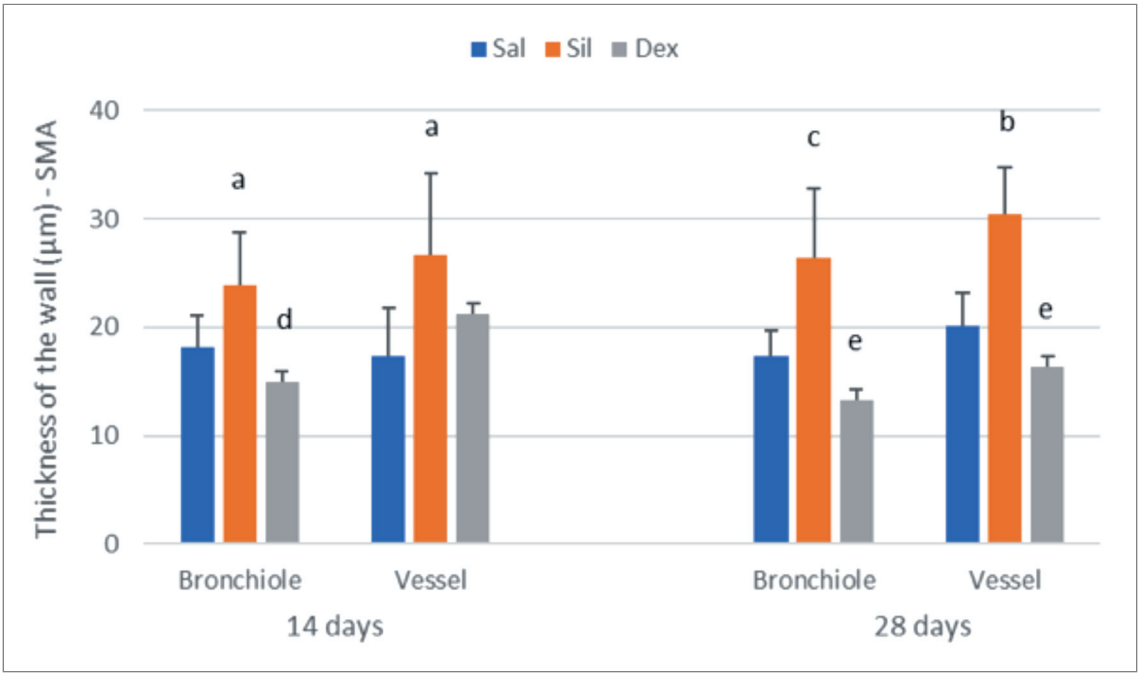


**Fig. 4.** Thickness of the wall of bronchioles and pulmonary vessels (in µm) – staining for presence of smooth muscle actin (SMA) by antibody against SMA in the control groups with saline instead of silica (Sal), in the silica-injected and non-treated animals (Sil), and in the silica-injected and dexamethasone-treated animals (Dex) after 14 or 28 days of the treatment delivery.

Statistical differences among the groups: [a] $p<0.05$ for Sil14 vs Sal14, [b] $p<0.001$ for Sil28 vs Sal28, [c] $p<0.01$ for Sil28 vs Sal28, [d] $p<0.01$ for Dex14 vs Sil14, [e] $p<0.001$ for Dex28 vs Sil28.

## DISCUSSION

Inhaled silica particles may interact with pulmonary epithelial cells and resident macrophages, but also with dendritic cells and other immune cells contributing to both innate and adaptive immune responses. Our pilot study demonstrated that significant mobilization of leukocytes and first fibrotic changes in the walls of bronchioles and pulmonary vessels can be found already 14 days after a silica instillation into the rat lung. Early administration of dexamethasone mitigated the above mentioned changes suggesting its potential use in the treatment of silicosis.

Inhaled silica immediately reacts with immune cells, which causes their activation and subsequent overproduction of ROS and massive generation of proinflammatory cytokines *via* an increased expression of transcription factors including nuclear factor (NF)-κB (3,13,14). Our results demonstrated that the inflammatory changes detected after the silica instillation were associated with an increased total count of leukocytes in the blood and elevated absolute numbers of circulating lymphocytes and slightly increased counts of neutrophils

and eosinophils. Higher absolute numbers of lymphocytes, neutrophils, and eosinophils were also found in the BAL fluid of the silica-instilled compared to the saline-instilled animals after both 14 and 28 days of the experiment. The decreased percentage of circulating monocytes in the blood of the silica-instilled animals compared to the saline-instilled controls could indicate that these cells migrated from the circulation into the injured lung. However, the percentage of macrophages in the BAL fluid even decreased compared to the saline-instilled group. This may indicate that a part of macrophages could be destroyed by a direct cytotoxic action of silica. The macrophages which survived their contact with extremely toxic silica can move to the lung interstitium where they can transform to activated interstitial macrophages and participate in the progression of lung fibrosis (15), which may partially explain their shortage in the alveolar compartment and lumen of the airways from which the BAL fluid is extracted.

On the other hand, significant increases in neutrophils, eosinophils, and lymphocytes in the BAL fluid were also observed by other authors (16-19). For instance, a significant increase in total cell counts, but particularly in neutrophil counts, in the BAL fluid together with an increased count of neutrophils in the blood was found already in the first days of silica exposure in the silica-aerosol induced model of silicosis (13).

A persistence of inflammation because of an ineffective clearance of phagocyted material and a release of macrophage-derived products is associated with activation of profibrotic mechanisms, which finally results into irreversible fibrotization of the lung tissue (2,18,19) and pathological changes in the airways (17). The comparison of the silica-instilled and the saline-instilled animals in our study showed a significant accumulation of collagen and smooth muscle mass in the walls of bronchioles and pulmonary vessels in the silica-injured animals already after 14 days of silica exposure and the extent of fibrotization enlarged during the following 14 days of the experiment. This finding is in agreement with the study of Porter et al. (13) who found a progressive worsening of the lung inflammation and fibrosis with increasing time after the silica exposure. Surprisingly, slight fibrotic changes were also detected in the saline-instilled animals after 14 days when compared to 28 days of the experiment. We can hypothesize that the orotracheal instillation of sterile saline in the control group could initiate some local inflammatory response resulting into a slight increase in collagen formation which was detected only by Sirius red staining, but not by antibody against SMA. Our results are in agreement with other authors who found a significant but transient increase in annexin A5, a marker of apoptosis (20), or sligh but non-significant increases in lactate dehydrogenase, a marker of cell injury, and protein content in BAL fluid (21), or elevations in hydroxyproline, a component of collagen, and malonyldialdehyde, a marker of lipid peroxidation in the lung tissue (22) of the saline-instilled animals.

The therapeutic action of corticosteroids is mediated by complex genomically- and non-genomically-mediated mechanisms (23,24). These mechanisms result into effective suppression of inflammation *via* modulation of inflammatory cascades on various levels and influencing various cells including respiratory epithelium, endothelium, macrophages, eosinophils, neutrophils, and lymphocytes, as well as into the reduction of oxidative and fibrotic changes (25-27). However, the effects of corticosteroids may be largely non-homogenous depending on the type, dose, delivery route, and timing of the given therapy, as we previously published elsewhere (28). In this study, early treatment with dexamethasone decreased the total count of circulating leukocytes, showed a clear trend to reduce the mobilization of inflammatory cells into the BAL fluid and prevented fibrotic changes in the lung as expressed by a decrease in production of collagen and lower accumulation of smooth muscle mass in the walls of bronchioles and vessels in comparison to the non-treated Sil group. In agreement with our results, partial alleviation of inflammatory infiltrate, granulomatous response, and generation of collagen after dexamethasone treatment was demonstrated in other two studies in silica-instilled mice (19, 29). However, in the study by Rabolli et al. (19) the

reduction in inflammatory response after dexamethasone was not associated with antifibrotic effect. Variability in the antifibrotic response to given dexamethasone may be related to inter-species differences of laboratory animals which were used for preparing the model of silicosis. Thus, dexamethasone may reduce the pulmonary fibrotic response in rats (30), but it may omit such effect in mice (31). The decreased markers of inflammation and oxidative stress, mitigated expression of NF-κB, and lower total counts and neutrophil counts in the BAL fluid after dexamethasone treatment was demonstrated by other authors in the silica-instilled animals (32,33) but also by us in the models of acute lung injury (34,35). Dexamethasone-induced inhibition of chemotactic factors may result in a reduction of neutrophil margination and influx into the lungs, with their simultaneous increase in the blood (36,37) as also demonstrated in our previous studies (34,35). On the other hand, the potential of dexamethasone to reduce the numbers of eosinophils, neutrophils and lymphocytes in the blood and BAL fluid shown in our study is in agreement with the action of corticosteroids in the treatment of other respiratory diseases including bronchial asthma (38-40), chronic obstructive pulmonary disease (41-43), community-aquired pneumonia (44), or various types of acute lung injury (45).

Of course, we are aware of several limitations of our experimental study. In this phase of the project, only male animals were used. Female animals are more sensitive to induction of various forms of lung fibrosis including silicosis, which may be associated with more frequent complications and higher mortality (46,47), therefore, a majority of animal studies with modeling of lung fibrosis or silicosis use male rats or mice. Moreover, dynamics of progression of pulmonary silicosis in patients inhaling silica particles in higher or smaller concentrations is gradual and develops within the years. In this context, the distribution of the silica particles after an artificial instillation in the laboratory animals is different. However, as demonstrated in many previous studies (2,13,14,19,31), silica induces rather homogenic local response in the immune cells and the silica-induced inflammatory and fibrotic changes in the lung of laboratory animals are fully comparable with the changes observed in patients suffering from lung silicosis. The other objection may arise from the use of corticosteroids in the treatment of silicosis regarding their wide adverse effects in long-term use. In this study, the long-acting corticosteroid dexamethasone was used for only 28 days to evaluate its potential in administration in an early phase of the development of silicosis. Our results showed that dexamethasone given early may prevent later development of massive inflammatory and fibrotic changes in the lung. These findings may subsequently provoke the following research regarding the early or even preventive use of any anti-inflammatory agents (either synthetically produced or natural compounds) which may prevent the development and progression of irreversible fibrotic changes after silica inhalation.

## CONCLUSIONS

Our pilot study has shown that early administration of dexamethasone exerted anti-inflammatory, anti-fibrotic and anti-remodeling effects in the rat model of lung silicosis suggesting a future perspective for the early use of anti-inflammatory drugs including corticosteroids in the treatment of lung fibrosis.

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# DIAGNOSIS AND CLASSIFICATION OF HEREDITARY FIBRINOGEN DISORDERS

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## Abstract

Hereditary fibrinogen disorders (HFDs) are rare bleeding disorders with a wide spectrum of biological and clinical features. While most patients with HFDs are at risk to suffer from mild to severe, sometimes life-threatening bleeding, thrombotic events are also common. Therefore, an appropriate diagnosis is needed to offer the optimal treatment. Diagnosis of HFDs can be challenging and plenty of pitfalls. The sensitivity and specificity of hemostasis routine test are depending on the reagents, the methods, and the fibrinogen variants. To distinguish subtypes of HFDs additional tests are often required. Historically based on the assessment of fibrinogen levels, a recent classification also considers the clinical phenotype and the genotype. In this short review, diagnosis strategies and HFDs classification are reviewed.

## INTRODUCTION

Hereditary fibrinogen disorders (HFDs) encompass a relatively large group of fibrinogen deficiency[1]. HFDs are classically subdivided into types 1 (afibrinogenemia and hypofibrinogenemia) and types 2 (dysfibrinogenemia and hypodysfibrinogenemia)[2]. Clinical features vary according to the fibrinogen level and the subtype of HFDs. Afibrinogenemia, characterised by the complete absence of fibrinogen, is typically associated with a severe bleeding phenotype and a paradoxical thrombotic tendency[3,4]. Hypofibrinogenemia, defined by decreased levels of fibrinogen activity and antigen, is associated with a bleeding risk dependent on the fibrinogen concentration[5,6]. In dysfibrinogenemia, normal levels of dysfunctional fibrinogen lead to highly heterogenous clinical features, from mild to life-threatening bleeding to recurrent thrombotic events, even though most patients are asymptomatic at time of diagnosis[7]. Hypodysfibrinogenemia, in which both activity and antigen fibrinogen levels are decreased, is often marked by a strong tendency to bleeding and thrombosis[4,8]. A recent classification identified several sub-types of HFDs considering fibrinogen activity and antigen levels, genotype, and clinical phenotype. Therefore, an accurate diagnosis is essential for the correct identification of patients and to optimize their management.

In this short review we will discuss the stepwise procedure leading to diagnosis, we will highlight some pitfalls, and we will give some details on classification of HFDs.

### Diagnosis of hereditary fibrinogen disorders

A fibrinogen disorder is usually suspected in patient addressed for a bleeding tendency with decreased fibrinogen level. However, incidental finding is frequent, especially in patients with mild fibrinogen deficiencies. Before starting investigations of HFD, it is important to rule out an acquired fibrinogen disorder. Liver disease, drugs and cancer, the most common causes, can affect the synthesis, the secretion, or the proteolysis of fibrinogen resulting in decreased levels of fibrinogen activity[9,10]. The familial history is also crucial, often helping to distinguish between a hereditary and acquired fibrinogen disorder.

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**Table 1** Biological features of hereditary fibrinogen disorders and International Society Thrombosis and Hemostasis (ISTH) classification

| | Fibrinogen activity | Fibrinogen antigen | Genotype | ISTH classification |
|---|---|---|---|---|
| **Afibrinogenemia** | No detectable | No detectable | Homozygous or compound heterozygous for a null* mutation | 1A. Bleeding phenotype or asymptomatic individuals<br>1B. Thrombotic phenotype |
| **Hypofibrinogenemia** | Decreased | Decreased | Heterozygous for a null or missense mutation | 2A. Fibrinogen activity <0.5 g/L (severe)<br>2B. Fibrinogen activity 0.5 - 0.9 g/L (moderate)<br>2C. Fibrinogen activity 1- and lower limit of normal value (mild)<br>2D. Histologically proven accumulation of fibrin in hepatocytes (Fibrinogen storage disease**) |
| **Dysfibrinogenemia** | Decreased*** | Normal*** | Heterozygous for a missense mutation | 3A. Bleeding phenotype or with thrombotic phenotype not fulfilling criteria for dysfibrinogenemia 3B or asymptomatic individuals<br>3B. Carriers of a thrombotic fibrinogen mutation**** or suffering from thrombotic events with a first-degree familial thrombotic history (relatives with the same genotype) without any other thrombophilia |
| **Hypodysfibrinogenemia** | Decreased | Decreased | Homozygous or compound heterozygosity for a null and/or a missense mutation | 4A. Fibrinogen antigen <0.5 g/L (severe)<br>4B. Fibrinogen antigen 0.5 - 0.9 g/L (moderate)<br>4C. Fibrinogen activity 1- and lower limit of normal value (mild) |

*: large deletions, splice-site mutations, frameshift mutations and nonsense mutations; **: Fibrinogen Brescia, Fibrinogen AI du Pont, Fibrinogen Pisa, Fibrinogen Ankara, Fibrinogen Angers, Fibrinogen Beograd, Fibrinogen Trabzon; ***: dependent on the reagents, the methods, and the fibrinogen variant; ****: Fibrinogen Dusart, Fibrinogen Caracas V, Fibrinogen Ijmuiden, Fibrinogen New York I, Fibrinogen Nijmegen, Fibrinogen Naples at homozygous state, Fibrinogen Melun.

The first step in diagnosis of HFDs relies on measurement of fibrinogen activity by the Clauss method[11]. As second step, if Clauss assay is decreased, fibrinogen antigen has to be measured. As indicated in table 1, if both activity and antigen are undetectable, afibrinogenemia can be diagnosed. If fibrinogen activity and antigen are proportionally decreased, a hypofibrinogenemia should be considered. Discordance between decreased fibrinogen activity and normal antigen is highly predictive of dysfibrinogenemia. A ratio fibrinogen activity / antigen with a cut-off of 0.7 has historically be proposed to distinguish between quantitative and qualitative fibrinogen disorders, but not fully validated[12]. Recently, a cut-off of 0.55 has also been tested[13]. If fibrinogen activity and antigen are disproportionally decreased, a hypodysfibrinogenemia should be considered.

Thrombin time and reptilase time are not mandatory for the diagnosis of HFDs, even though they provide useful information on the degree of defect in fibrin polymerisation[14]. Derived fibrinogen from the prothrombin time (PT-der) is widely used to assess the fibrinogen concentration[15]. The PT-der is an indirect estimation of fibrinogen concentration. In dysfibrinogenemia, PT-der overestimated the fibrinogen activity but has a very good correlation with antigen value[16]. A ratio PT-der / Clauss with a cut-off of 1.43 seems to provide an excellent specificity and sensitivity for the diagnosis of dysfibrinogenemia[17]. In addition, recent data promotes novel approaches for diagnosis of HFDs. Clot wave form from Clauss could have a similar performance to detect dysfibrinogenemia[13].

It should be noted that in dysfibrinogenemia several variables such as clot detection methods, reagents, and type of fibrinogen variant, influence the measurement of routine hemostasis assays[18]. For instance, Fibrinogen Longmont is better detected by photo-optical endpoint[19,20]. In case of very low values of fibrinogen activity, due the limit of quantification of analysers, it could be difficult to distinguish between afibrinogenemia and hypofibrinogenemia or between hypofibrinogenemia and hypodysfibrinogenemia. In these cases, genotype is necessary to confirm the diagnosis[21]. In the setting of research, analyses of fibrin clots, may complete the fibrinogen work-up in order to better determine the patient's clinical phenotype[22].

**Molecular analysis**

HFDs result from monoallelic or biallelic mutations in *FGA*, *FGB* and *FGG* genes in chromosome 4[23]. Besides the confirmation of the diagnosis, genotype may help for familial screening, prenatal testing, and prediction of the clinical phenotype. The development of next generation sequencing allows complete analysis of *FGA*, *FGB* and *FGG* exons enhancing the identification of a causative mutation[24]. However, in developing country, Sanger sequencing is still the best option[25]. Usually, afibrinogenemia results by homozygosity for a null mutation[26]. Thus, patients with hypofibrinogenemia are heterozygous carriers of afibrinogenemic allele. These mutations affect either the synthesis or the assembly or the secretion of fibrinogen into circulation. Some mutations are more common, such as the large deletion 11Kb of *FGA* and the splice site mutation *FGA* c.510+1G>T[27,28]. Most of dysfibrinogenemia are due to missenses mutation located in exon 2 of *FGA* or exon 8 of *FGG*[29]. Overall, two hotspot mutations (i.e, *FGA* c.103C>A or c.104G>A and *FGG* c.901C>T or c.902G>A) represent more than 80% of causative mutations in dysfibrinogenemia. These mutations yield to a delay in thrombin-mediated fibrinopeptide cleavage or defective fibrin polymerisation[30,31]. In hypodysfibrinogenemia, a single mutation can affect both the secretion and the function of the fibrinogen molecule. In alternative, a compound mutation can confer a concomitant "hypofibrinogenemia" and "dysfibrinogenemia" trait[4].

A few fibrinogen variants are strongly correlated with a clinical phenotype[32]. Patients with thrombotic-related fibrinogen variants are a very strong risk of thrombosis[33]. Thrombosis

can occur in all vascular territories, including in young patients[34-39]. These mutations affect the fibrin clot structure leading to a procoagulant state[40]. Other mutations, located in exons 8 and 9 of *FGG* cause accumulation of fibrinogen aggregates in hepatocytes resulting in chronic liver inflammation and eventually cirrhosis[41]. In dysfibrinogenemia, mutations modifying specific aspects of the fibrinogen molecule, can decrease the clottability and therefore increase the bleeding tendency. For instance, mutations in the NH2-terminal portion slow the conversion of fibrinogen into monomeric fibrin; mutations adding new N-linked glycosylation sites introduce negatively charged carbohydrate side chains affecting the alignment of fibrin monomers during polymerization; mutations generating unpaired cysteine form extra disulfide bonds and produce highly branched and fragile fibrin networks; truncation mutations in the fibrinogen αC regions impair the lateral fibril aggregation and factor XIII crosslinking[30].

## Classification of hereditary fibrinogen disorders

As indicated in Table 1, the International Society Thrombosis and Hemostasis (ISTH) classification introduces several subtypes of HFDs according not only on the fibrinogen levels but also on the clinical pattern and the genotype. Although severe bleeding is the prominent symptom in afibrinogenemia, some patients experience recurrent thrombosis[42]. In a recent large series of patients (n=204), 18% reported a thrombotic event[43]. Management of thrombosis in afibrinogenemia is particularly difficult as physician has to deal with both the thrombotic and the bleeding risk[6]. Patients with afibrinogenemia and a thrombotic phenotype are classified as type 2A.

The bleeding risk in hypofibrinogenemia is strongly correlated to the fibrinogen concentration. In a registry from the Netherlands, the correlation between baseline fibrinogen activity and ISTH bleeding assessment tool score was considered as moderate (r= -0.683)[44]. It is generally well accepted that patients with fibrinogen activity levels >0.8 g/L do not suffer from spontaneous bleeding[45]. Therefore, patients with hypofibrinogenemia are classified as severe (2A), moderate (2B), or mild (2C) according to the fibrinogen activity. A fourth subtype (2D) is hypofibrinogenemia associated with fibrinogen storage disease. This subtype is classically suspected in familial history of mild hypofibrinogenemia and cryptogenic liver disease. The physiopathology is not fully understood. Mutations in fibrinogen γ chain could provoke conformational changes resulting in abnormal exposure of hydrophobic patches that become available for interactions with APOB-lipoproteinemia causing their intracellular retention and impairment of fibrinogen secretion[46].

Most of patients with dysfibrinogenemia will be asymptomatic at the time of diagnosis[47-49] but the natural course of dysfibrinogenemia is nevertheless characterized by a risk of bleeding and thrombosis. In a series of 101 patients with dysfibrinogenemia with a median follow-up of 8.8 years, the cumulative incidence of major bleeding and thrombotic events was 2.5 and 18.7 per 1000 patient-years, respectively, with estimated cumulative incidences at age 50 years of 19.2% and 30.1%[7]. These patients are classified as subtype 3A. As previously mentioned, the thrombotic risk is remarkably more important in patients with thrombotic-related fibrinogen variants (subtype 3B). In such patients, thrombosis usually occur in young patients, including in unusual vascular sites.[50]

In hypodysfibrinogenemia the bleeding risk is proportional to the fibrinogen level, but it is also exacerbated by the dysfunctional fibrinogen. Indeed, hypodysfibrinogenemia is characterized by reduced fibrinogen function but also a less marked reduction of plasma fibrinogen antigen[8]. Therefore, patients with hypodysfibrinogenemia are classified as severe (2A), moderate (2B), or mild (2C) according to the fibrinogen antigen.

## CONCLUSIONS

Diagnosis of HFDs is a stepwise procedure. Development and/or integration of alternative techniques to assess the fibrinogen concentration could help in the next future to minimise diagnosis incertitude. A better knowledge of the molecular basis and their correlations with the clinical phenotype will allow to a more subtle classification of HFDs.

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# PAROXYSMAL NOCTURNAL HEMOGLOBINURIA: A RARE CASE OF RECURRENT EPISODES OF ICTERUS

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## Abstract

In this work we describe a rare case of a patient with paroxysmal nocturnal hemoglobinuria (PNH) developed on the background of specific medical history of morbus Gilbert with chronically elevated bilirubin and recurrent attacks of macroscopic haematuria. We focus on a differential diagnosis of the disease, its obstacles, and treatment options.

**Key words:** paroxysmal nocturnal hemoglobinuria, haemolytic anaemia, icterus

## INTRODUCTION

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare acquired clonal disease of hematopoetic stem cell, without malignant potential. PNH is caused by a somatic mutation of genes on X chromosome, usually PIGA gene is involved. These genes are responsible for anchoring of several proteins such as CD55 and CD59 on the surface of blood cells. These mutations could lead to the production of deficient blood cells with a decreased or totally absent resistance to complement, which leads to the episodes of haemolysis. PNH is a potentially life threating disease associated with intravascular and frequently also with extravascular haemolysis, thromboembolic events, and in some cases with myelodysplastic syndrome (MDS) or aplastic anaemia AA (1).

The main mechanism in pathophysiology of PNH is a deficiency of CD55 and or CD59 on the surface of blood cells. The main purpose of these proteins is to inhibit complement activation on membrane surface. In the case of decreased or absent complement inhibition, the state of complement overactivation is achieved, which leads to intravascular haemolysis that gives rise to a leak of haemoglobin to circulation (2). Free haemoglobin has a toxic effect on endothelium, which precipitates thrombosis formation. The exact mechanism is a subject of an ongoing debate and research. The other pathophysiological mechanism in PNH is caused by free circulating fragments of activated complement which accumulate in reticuloendothelial system in spleen and liver. This leads to a further damage of these organs.

Patients usually manifest with nonspecific signs like fatigue, lethargy, dyspnoea, headaches, chest pain, abdominal pain, and odynophagia. Other symptoms are jaundice, haemoglobinuria, and thrombosis in atypical locations such as splanchnic veins, cranial venous sinuses, and skin veins (table number 1).

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**Table 1** Frequency of symptoms in patients with proximal nocturnal hemoglobinuria

| Symptoms | Frequency |
|---|---|
| Fatigue | 80% |
| Dyspnoea | 64% |
| Headache | 63% |
| Hemoglobinuria | 62% |
| Icterus | 50% |
| Abdominal pain | 50% |
| Erective dysfunction | 38% |
| Chest pain | 33% |
| Dysphagia | 25% |

In laboratory tests we detected signs of intravascular haemolysis, iron deficiency, leukopenia, thrombocytopenia, rise of creatine, and hepatopathy (3). Indication for screening for paroxysmal nocturnal hemoglobinuria are shown in table number 2.

**Table 2** Indication for screening for paroxysmal nocturnal hemoglobinuria

| |
|---|
| 1. Chronic Coombs negative intravascular haemolysis with LD elevated above 1.5 UNL, decreased haptoglobin or kidney injury |
| 2. Thrombosis in atypical location, or thrombosis in a young patient without other explanation and sings of haemolysis |
| 3. Dysphagia, abdominal pain, erective dysfunction and sing of haemolysis |
| 4. Signs of haemolysis and iron deficiency |
| 5. Patient with aplastic anaemia or myelodysplastic syndrome |
| 6. Idiopathic cytopenia without other explanation |

We recognize three main groups of PNH. A classic PNH where dominant clinical presentation is haemolysis and thrombosis with minimal bone marrow involvement. Patients with the classic form of PNH usually have significant PNH clone population. A subclinical PNH which is characterized by a lack of clinical symptoms and laboratory signs of haemolysis but there is a detectable population of PNH clone by flow cytometry. And the last group is PNH developed in context of other disease with bone marrow failure like myelodysplastic syndrome PNH/MDS or aplastic anaemia PNH/AA (4).

The therapeutic approach is mostly determined by a clinical form of disease. In case of the classic PNH, treatment is based on a target therapy and supportive care like B12, B9, and iron substitution. An important part of management of PNH is also prevention of complement stimulation by preventing infectious diseases with vaccinations and early antibiotics treatment (5). The most effective treatment is a target therapy with anti C5 monoclonal antibodies with eculizumab. This approach blocks out C5 part of complement cascade which leads to the

prevention of MAC (membrane attacking complex) formation. This leads in averting further haemolysis in most patients with PNH (6). A small subgroup of patients is only a partial responder or no responder. This can happen either due to the resistance of C5 to eculizumab or dysregulation on C3 level of complement. These patients could benefit from antibodies targeted to C3, such as pegcetacoplan (7). Adverse effects of treatment with anti C5 antibodies include an increased risk of an infection, especially meningococcal infections. This is the reason why it is highly recommended for all patients on eculizumab to be vaccinated.

The main cause of mortality and morbidity in patients with PNH are thromboembolic events. In case of past thromboembolic event some authors do recommend lifelong anticoagulation therapy. There are no data comparing efficacy of low molecular heparin with direct oral anticoagulant or warfarin. In case of an acute thromboembolic event, the regular management according to location is recommended (8).

## CASE REPORT

In this article we presented the case of a 27-year-old male with a medical history of recurrent attacks of haematuria, jaundice, and transitory elevations of liver enzymes.

The first episode that led to a hospitalization occurred in October 2019. The patient was hospitalized in Sweden with an attack of haematuria, jaundice, and elevations of liver enzymes. This attack was interpreted as a non-severe case of acute alcoholic hepatitis. Later that year the patient was re-hospitalized in Slovakia with yet another attack with a similar clinical manifestation. This time the clinical interpretation was a drug induced liver injury with an acute kidney injury. After discharge the patient was referred to a nephrologist and a gastroenterologist for further management.

In June 2020 the patient was once again hospitalized at department of Internal Gastroenterological Medicine with another attack. Main clinical manifestation was haematuria jaundice pain in the lumbar region. The patient did report recurrence of this pain usually followed by a period with dark coloured urine. Typically, this conditions spontaneously disappear in two to three days. Laboratory findings were dominated by normocytic normochromic anaemia, thrombocytopenia, hyperbilirubinemia, elevation of lactate dehydrogenase, and decrease of haptoglobin. For overall laboratory results see table number 3. Urine aseptic haematuria was detected but there was no detection of erythrocytes in microscopic analysis.

**Table 3** Laboratory result during attack of haemolysis

| Laboratory result | |
|---|---|
| Haemoglobin | 130 (g/L) |
| MCV | 98.9 (fL) |
| Reticulocyte absolute count | 225.9 (1012/L) |
| Reticulocyte relative count | 5.7 (%) |
| Ferritin | 275.30 (µg/l) |
| Lactate dehydrogenase | 68 (µkat/L) |
| Haptoglobin | 0.08 (g/l) |
| Direct bilirubin | 81.1 (µmol/l) |
| Indirect bilirubin | 9.5 (µmol/l) |

The differential diagnosis of haemolytic anaemia was initiated based on the clinical picture. Coombs tests were negative, blood schwab was without a presence of schistocytes or changes in shape of erythrocytes. ADAMTS 13 activity was examined to definitively rule out thrombotic thrombocytopenic purpura.There was no significant decrease in enzyme activity.

In the next step a flow cytometry was performed where a population of PNH cells was detected (see Fig. 1 and Fig. 2).

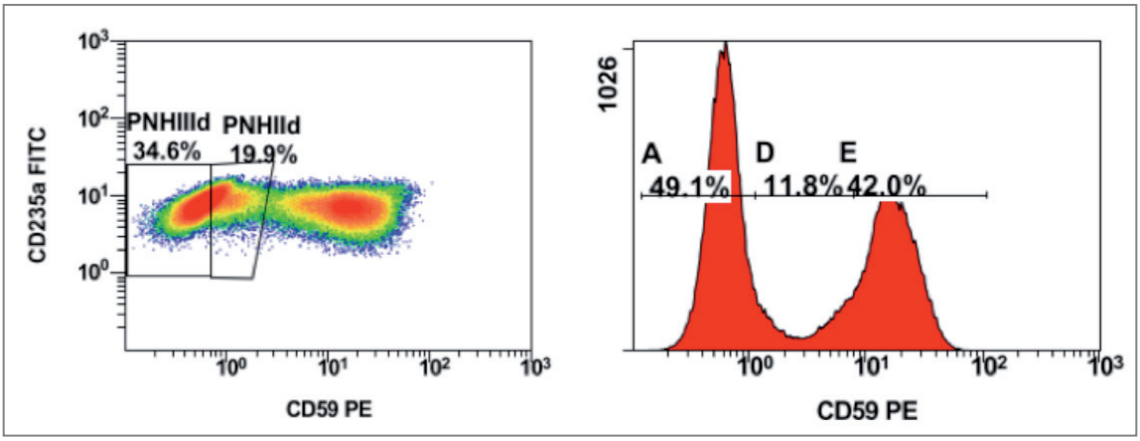


**Fig. 1** Erythrocyte population with phenotype typical for paroxysmal nocturnal haemoglobinuria

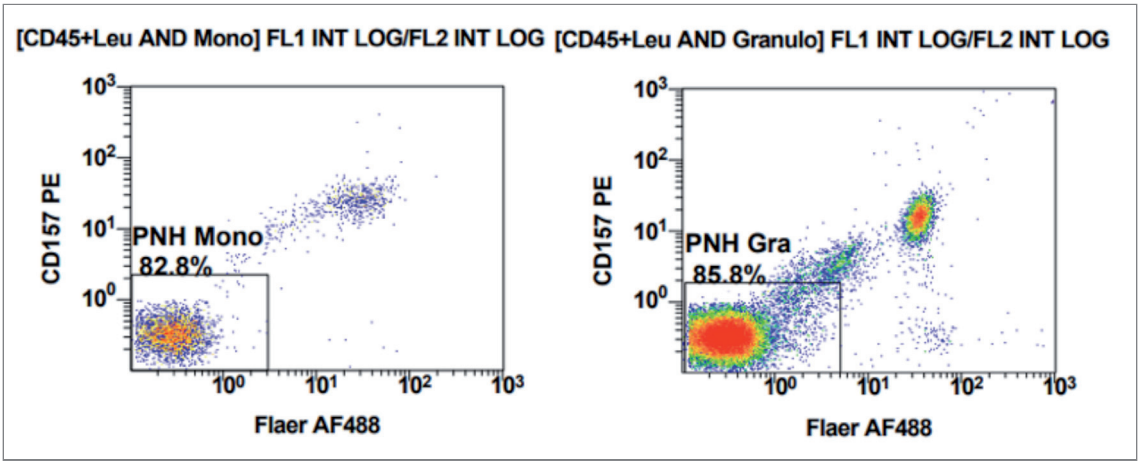


**Fig. 2** Population of monocytes and granulocytes with phenotype typical for paroxysmal nocturnal haemoglobinuria

Due to the presence of thrombocytopenia in cooperation with haematologist we performed a bone marrow biopsy to rule out myeloblastic syndrome or aplastic anaemia. No specific signs of dyshaematopoesis were detected in the biopsy. During the hospitalization, *Stafylococcus aureus* was detected by a cultivation from nasopharyngeal schwab. This was interpreted as a precipitating factor for an attack of haemolysis, subsequently a target antibiotic therapy was initiated, and after a period of observation the patient was discharged. Afterward, the patient was referred for ambulatory care by a haematologist, where a therapy with eculizumab was started. On this therapy up to date there has been no major throm-

boembolic event or a need for hospitalization or hemosubstitution. In a periodical laboratory check, we observed a stable hemogram but oscillations in lactate dehydrogenases, bilirubin, and reticulocytes suggesting remaining inconsequential level of haemolysis (Tab 4). There was no significant shift in PNH population in a flow cytometry (Tab 5).

**Table 4** The oscillation of biochemical parameter after eculizumab treatment

| Date | TBIL (μmol/l) | AST (μkat/l) | LD (μkat/l) | ERY ($10^{12}$/L) | RET ($10^{12}$/L) |
|---|---|---|---|---|---|
| 12/01/21 | 84.6 | 0.44 | 4.76 | 4.45 | 369 |
| 04/03/21 | 67.9 | 2.36 | 24.98 | 4.09 | 185.7 |
| 16/03/21 | 61.2 | 0.52 | 4.64 | 4.45 | 239.2 |
| 06/07/21 | 54.2 | 0.81 | 7.66 | 3.8 | 363.5 |
| 16/09/21 | 90.9 | 0.48 | 3.79 | 4.55 | 232.9 |
| 09/12/21 | 79.1 | 1.96 | 13.93 | 4.87 | 194.3 |

**Table 5** Evolution of PNH population before and after treatment with eculizumab

| PNH population | 27/10/20 | 16/3/21 | 23/11/21 |
|---|---|---|---|
| Granulocyte | 82.8% | 85.3% | 85.5% |
| Monocyte | 78.6% | 83.6% | 82.7% |
| Erythrocyte | 55.2% | 57.4% | 55.4% |

## DISCUSSION

In our case report we focus on struggle and obstacles of diagnosis of PNH, especially if this disease is present in a patient with comorbidities masking the main symptoms of haemolysis. In our case it was *morbus Gilbert*. As described in our case report, this led to repeated misdiagnoses and quite significant prolongation in the diagnostic time.

PNH is a rare potentially life-threatening disease with a very low prevalence in population. Epidemiological data from United Kingdom suggest an incidence of 3.5 cases per million people. Because of broad clinical manifestation and inexperience of clinicians with PNH the time from the first manifestation to the diagnosis could be significantly prolonged as was seen in our case report. In some cases, the first manifestation could be severe thromboembolic event with possibly fatal consequences. The prognosis significantly varies for each subgroup. In general, five-year survival rate is 72% for all types of PNH (9).

The diagnosis is based on medical history, clinical manifestation of Coombs negative haemolytic anaemia, for definitive diagnosis flow cytometry is performed. This method can unarguably confirm the presence of PNH clone in peripheral blood (10). Furthermore, the size of individual clone population for granulocytes and erythrocytes can be count. This is quite useful since the size of erythrocyte PNH clone is often underestimated. This usually happens because of extensive haemolysis and hemosubstitution. In contrast, the population of polymorphonuclears is not influenced by the deficit in surface CD55/59, that is why

polymorphonuclears clone size corelates with the extension of the disease more precisely (11). The flow cytometry is a relatively widely available and cheap diagnostic tool and should be performed whenever PNH is suspected. The indication for flow cytometry could be found in table no 2. During the diagnosis the other cause of Coombs negative haemolytic anaemia should be considered (see table 6).

**Table 6** Differential diagnosis of paroxysmal nocturnal haemoglobinuria

1. Haemolytic anaemia
   - 1.1. Autoimmune haemolytic anaemia
   - 1.2. Drugs induce haemolytic anaemia
   - 1.3. Intoxication
   - *1.4. Enzymopathies*
     - 1.4.1. G6PD deficiency
     - 1.4.2. Pyruvate kinase deficiency
   - 1.5. Hemoglobinopathies
     - 1.5.1. Sickle cell anaemia
     - 1.5.2. Thalassemia
   - 1.6. Membranopaties
     - 1.6.1. Spherocytosis
     - 1.6.2. Elliptocytoses
   - 1.7. Infections (malaria, HIV)
   - 1.8. Microangiopathies
     - 1.8.1. TTP
     - 1.8.2. HUS
     - 1.8.3. HELLP
     - 1.8.4. Uremic syndrome
     - 1.8.5. DIC
     - 1.8.6. Drug induced macroangiopathic haemolytic anaemia
   - 1.9. Systemic diseases
     - 1.9.1. Systemic Lupus erythematosus
     - 1.9.2. Vasculitis
2. Thromboembolic events with atypical localized thrombus
3. Bone marrow diseases
   - 3.1. Myelodysplastic syndrome
   - 3.2. Aplastic anaemia

In the case of our patient, we observed severe prolongation from manifestation to the diagnosis of PNH. In the period before the initiation of eculizumab administration the patient had significantly increased the risk of development of severe haemolysis or thromboembolic events with potentially fatal consequences. For these reasons a prompt diagnosis and treatment is crucial in all cases of PNH.

## CONCLUSION

PNH is a rare disease, with a broad spectrum of clinical manifestation and challenging differential diagnosis. Many clinicians struggle with prompt diagnosis of PNH. This could get even trickier if PNH developed in a context of other disease such as Gilbert disease. Due to the association of PNH with potentially life-threatening thromboembolic complications the early diagnosis and proper management is crucial for prognosis of each patient.

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# A CORRELATIVE INTERACTION BETWEEN THYROID DYSFUNCTION AND SEMEN PARAMETERS IN MALE INFERTILITY: A PROSPECTIVE CASE CONTROL STUDY

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## Abstract

The role of thyroid function on sperm quality has not been well studied from a pathological aspect. This study aimed to report the degree of association between the status of thyroid hormones, sperm quality and aetiology in infertile men compared to healthy subjects. A prospective case control investigative study was conducted on 100 infertile males and age matched healthy controls. Semen samples were collected for sperm quality examination, and the serum levels of tetraiodothyronine (T4), triiodothyronine (T3), and thyroid stimulation hormone (TSH) were measured. Out of 100 infertile men, oligozoospermia (32%), asthenozoospermia (48%), and oligo-asthenozoospermia (20%) were found. There was a statistical difference between the group I and group II groups related to sperm count (28.32 ± 14.60 vs 66.50 ± 10.50 x $10^6$/ml), sperm motility (40.1 ± 13.8 vs 64.8 ± 7.85%), and sperm morphology (55.92 ± 5.27 vs 83.50 ± 5.25%, $p<0.05$). There was a statistical difference among the oligozoospermia, asthenozoospermia, and oligo-asthenozoospermia groups related to T3 (115 ± 0.40 vs 1.29 ± 0.59 vs 1.25 ± 0.32 ng/ml), T4 (7.35 ± 1.42 vs 9.15 ± 1.85 vs 7.85 ± 1.65 µg/dl), and TSH (1.69 ± 0.55 vs 2.12 ± 1.45 vs 1.98 ± 0.4 µIU/ml) ($P<0.05$). There was a significant inverse correlation of TSH levels with sperm volume ($r = -0.12$, $p= 0.02$), sperm motility ($r = -0.26$, $p= 0.02$), and sperm morphology ($r = -0.304$, $p = 0.02$) observed. T4 levels were significantly correlated with sperm count ($r = -0.278$, $p = 0.02$), and sperm motility ($r = -0.249$, $p = 0.032$). T4 levels were very highly associated with asthenozoospermia. Relative operating curve analysis shows that Sperm motility of >40.1%, T3 levels of <1.29 ng/ml, total T4 levels of <8.42 µg/dl, TSH levels of <1.98 µIU/ml inferred the male infertility.

**Conclusion:** Although thyroid function screening is not currently recommended as a part of the diagnostic workup of the infertile male, it may be reconsidered in light of the physiopathological background. Studies will be necessary to initiate the trial of a small dose of anti-thyroid drug in asthenozoospermic patients.

**Keywords**: Male infertility, Thyroid hormones, Oligozoospermia, Asthenozoospermia.

## INTRODUCTION

Infertility estimates ~15% of couples globally. Males are found to be solely responsible for 20-30% of infertility cases, and contribute to 50% of all cases (1). According to the Indian Society of Assisted Reproduction, infertility affects 10 to 14% of the Indian population, with higher rates in urban areas (2). In India, nearly 27.5 million couples are actively trying to conceive and suffer from infertility (3). According to the National Health Portal of India, 5% of infertility seen in Andhra Pradesh state and 15% of infertility in Kashmir state (4, 5).

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Previous research shows that a significant proportion of male infertility is idiopathic, and the underlying molecular mechanisms is still unknown (6). The significant factors of male infertility are low sperm concentration (oligospermia), poor sperm motility (asthenospermia), and abnormal sperm morphology (teratospermia), and other less associated male infertility factors are semen volume and seminal markers of epididymal, prostatic, and seminal vesicle function (7, 8). Although several causes of male infertility have been identified (9), the exact aetiology and pathogenesis of male infertility remain unknown (10, 11). Infertility aetiology varies from region to region, population to population, and even locality to locality within the same population (12).

Although the effects of hyperthyroidism and hypothyroidism on female reproduction are well established, the effects of thyroid disorders on male infertility have not been extensively studied, most likely because attention in thyrotoxic males is usually focused on other manifestations of the disease, and fertility status is frequently not evaluated (13,14). Thyroid hormones interfere with both androgen biosynthesis and spermatogenesis, either directly on Leydig and Sertoli cells or indirectly by modulating gonadotropin secretion (15). Thyroid hormones act on various cells of the male reproductive system via genomic and nongenomic mechanisms to regulate testicular testosterone secretion and the concentration of seminal plasma components such as calcium, fructose, magnesium, zinc, etc.

The optimal concentration of intratesticular testosterone promotes spermatogenesis, while other seminal properties improve sperm motility and viability and keep the sperm volume stable (16). Thyrotoxicosis can cause oligozoospermia, asthenozoospermia, and/or teratozoospermia, as well as decreased sperm volume (17). Hypothyroidism has also been associated with a decrease in serum testosterone levels. Hence, it is associated to erectile dysfunction, delayed ejaculation, hypoactive sexual desire, and poor sperm quality (18).

Hence, developing an effective preventive therapeutic intervention for male infertility necessitates a thorough understanding of the relationship between thyroid function and sperm quality. Extensive research is required to reveal the role of thyroid function and its disorders in the maintenance and deterioration of male infertility and to draw more reliable conclusions that can be used in clinical practise.

Hence, the current study investigated the relationship between thyroid hormone levels and sperm quality in a population of men recruited from an infertility clinic to detect associations between thyroid hormone levels and sperm quality and also with aetiology.

## METHODS

A prospective case control investigative clinical Study conducted from September 2019 to September 2022 at the department of Anatomy, Narayana Medical College, Nellore, Andhra Pradesh.

### Sampling Technique and Sample Size

The current study was carried out by a random sampling technique selecting the infertile males those were attending to the fertility clinic.

The sample size calculation was done by formula,:

Sample size (n) = $Z^2$ PQ/ $d^2$

Where, $Z^2$ value is fixed at 95% of confidence interval was 1.96,.

Prevalence (P) = 7%, i.e., 0.07 (based on previous studies)

Q = 1-P.

Error (d) is fixed at 5%., i.e., 0.05.

The sample size (n) = (1.96*1.96) *(0.07) *(0.93) / (0.05*0.05)

= 0.2501 / 0.0025 = 100.04 => 100.

Therefore, the sample size = 100.

A total of 100 group I (male infertility) cases, and an age matched 100 group II subjects (fertile male) were enrolled in the study. The Institutional Ethics Committee, Narayana Medical College, Nellore, India; an approval has been taken for the study protocol. A written informed consent form was taken from all the study subjects.

**The study groups:**

Group I (case) – Infertile males who fits into the inclusion criteria.

Group II (control) – Age matched fertile males without any co-morbidities.

**Inclusion criteria**

- Males with >24 years of age having no children for more than 2 years; even with unprotected sex and without achieving pregnancy.
- Physically and Mentally healthy males.
- Males without any congenital anomalies and co-morbidities.

**Exclusion criteria**

- Males with prostate cancer.
- Males with erectile dysfunction.
- Males with venereal diseases.
- Males with HIV.
- Psychologically unhealthy males.
- Males with co-morbidities like diabetes (both I & II), renal pathology.
- Patients with congenital anomalies of genital organ like hypospadias.
- Klinefelters syndrome.
- Hypogondotropic hypogonadism.
- Y-chromosome microdeletion or abnormality.
- Obstructive azoospermia.
- History of treatment with Cytotoxic drugs, irradiation, or sulfasalazopyrine.
- Patients who refuse to participate in the study.

**Data collection**:

Interviews for medical and nutritional history were part of the participant's examination.

**Semen analysis:**

After the detailed explanation about the procedure, the subjects were instructed to refrain from ejaculating for at least 72 hours before providing a sample of sperm. Masturbation in a clean wide mouth plastic container provided by the laboratory yielded the entire sample. The container was labelled with the patient's details, and the time and date of collection. The sample was liquefied for at least 20 minutes, but no more than 1 hour before performing a routine semen analysis, which included volume, pH, sperm concentration, sperm motility, progressive motility, and sperm morphology measurements. A fructose biochemical test was also performed. Sperm count performed using a Neubauer counting chamber and stained with Field's stain to check the sperm morphology.

Motile sperm was defined according to WHO grade as 'A' grade sperm (rapidly progressive with a velocity >25 μm/s at 37°C) and 'B' grade sperm (slow/sluggish progressive with a velocity >5 μm/s but <25 μm/s) (19).

Method for sperm motility: Morphological evaluation was carried out using an Olympus microscope and oil immersion. A coverslip was covered after placing a drop of sperm on the glass slide and examined under high power (40 ×) field. Motile spermatozoa were counted out of the 100. The percentages of motile and nonmotile spermatozoa were recorded.

Method of Sperm count: Semen was diluted 1:20 with formalin (0.1 ml semen and 1.9 ml formalin) and added the Neubauer++ chamber with diluted semen and allowed to settle for 15 minutes. The chamber was placed under the microscope and spermatozoa counted in 4 large corners squares using either 20 × or 40 × objective.

A drop of semen was placed on a glass slide, and prepared a smear, and stained the smear with field stain. Approximately 200 spermatozoa were counted under oil immersion. The percentage of normal & abnormal spermatozoa was recorded (20).

Sperm count per ml was calculated as follows:

$$= \frac{\text{Sperms counted (N)} \times \text{correction factor for dilution (20)}}{\text{Number of squares counted (4)} \times \text{volume of one square (0.1)}} \times 1000$$

$$= N \times 50000/\text{ml}.$$

On the same day of the sperm sample was collected, a blood sample was drawn using an aseptic technique after a 12-hour overnight fast. Sera were extracted from the blood samples. Total T3, T4, and TSH levels were assessed using Chemiluminescence Immunoassay (CLIA) assay.

**Statistics:**

The data was entered into a MS-Excel, and the statistics performed using the statistical package for Social Science Program (SPSS, IBM, US) for Windows Version 25.0. The data values for categorical variables were expressed as numbers and percentages, and the chi-square test were used to test the association between the groups. The data values for the continuous variables were expressed as mean and standard deviation, and to test mean differences between the two groups, the student's t-test was used. One-way analysis of variance (ANOVA) test was used to test mean differences among three or more groups. Pearson's correlation analysis was used to test the relationships between the parameters. All p-values less than 0.05 are considered statistically significant.

## RESULTS

The Group I consists 100 men with a mean age of 32.4 ± 4.5 years (range: 25- 40 years), and the group II comprised of 100 men with a mean age of 31.5 ± 4.8 years (range: 25 to 40 years).

In the group I, sperm counts varied from 9 to 60 million/ml with a mean of 28.32 ± 14.60, the percentage of sperm motility varied from 15 to 70 with a mean of 40.1 ± 13.8, and the percentage of normal sperm morphology varied from 40 to 70 with a mean of 55.92 ± 5.27, Whereas in the group II, the percentage of sperm counts varied from varied from 45 to 80 million/ml with a mean of 66.5 ± 10.5, the percentage of sperm motility with a mean of 64.8 ± 7.85, and the percentage of normal sperm morphology with a mean of 83.5 ± 5.25 with a statistical difference in sperm quality between the two groups ($p<0.05$) (Table 1).

Out of 100 infertile male, oligozoospermia was observed in 32 cases (32%, 95% confidence interval [CI], 27.5% - 35.5%), asthenozoospermia in 48 cases (48%, 95% confidence interval [CI], 41.5% - 50.5%), and oligo-asthenozoospermia in 20 men respectively (20%, 95% confidence interval [CI], 16.85% - 22.8%).

Total $T_3$ of oligozoospermic men ranged from 0.60 to 1.61 with mean of 115 ± 0.4, total $T_4$ varied from 5.5 to 9.1 with a mean of 7.35 ± 1.42, and TSH (µIU/ml) ranged from 0.89 to 2.65 with a mean of 1.69 ± 0.55.

Whereas in asthenozoospermic men total $T_3$ ranged from 0.72 to 2.65 ng/ml with a mean of 1.29 ± 0.59, total $T_4$ varied from 6.98 to 12.8 with the mean of 9.15 ± 1.85, and TSH (µIU/ml) ranged from 1.1 to 6.75 with a mean of 2.12 ± 1.45. Only total T4 was significantly higher in asthenozoospermic men compared to those with normal sperm profiles ($P<0.05$), but it was still well within the normal limits.

Total $T_3$ (ng/ml) of men with oligo-asthenozoospermia ranged from 1.12 to 1.70 with a mean of 1.25 ± 0.32, total $T_4$ varied from 5.5 to 10.5 with the mean of 7.85 ± 1.65, and TSH ranged from 1.60 to 2.20 with a mean of 1.88 ± 0.4.

After adjusting for potential confounders, the study revealed no significant differences in sperm volume, concentration, progressive sperm motility, or morphology between the euthyroid and SCH groups ($p>0.05$) (Table 2) (Table 3).

**Table 1** Comparison of semen and thyroid profile of normal and abnormal subjects

| Characteristics | | Group I (n=100) | Group II (n=100) | P value |
|---|---|---|---|---|
| Age (Years) | Mean ± SD | 32.4 ± 4.5 | 31.5 ± 4.8 | |
| Age (yr) | 21–25 | 10% | 10% | 0.15 |
| | 26–30 | 56% | 58% | |
| | 31–35 | 26% | 23% | |
| | 36–40 | 8% | 9% | |
| Duration of active married life (yr) | 2–3 | 35 | 34 | 0.26 |
| | 3.1–5 | 33 | 30 | |
| | 5.1–7 | 20 | 18 | |
| | 7.1–9 | 8 | 10 | |
| | 9.1–15 | 4 | 8 | |
| Semen analysis : Quantity (c.c.) | <1 | 16 | 2 | 0.0001* |
| | 2 | 57 | 63 | |
| | 2.1-3 | 16 | 15 | |
| | >3 | 12 | 20 | |
| Semen analysis : Sperm density (million/ml) | >15 | 48 | 100 | - |
| | 10.1 – 15 | 29 | 0 | - |
| | <10 | 23 | 0 | - |
| Semen analysis : Sperm motility | Actively motile | 32 | 100 | - |
| | Sluggishly motile | 40 | 0 | - |
| | Non-motile | 28 | 0 | - |
| Semen analysis : Frequency of motile sperm % | >50 | 22 | 100 | - |
| | 20.1-50 | 48 | 0 | - |
| | 5-20 | 30 | 0 | - |
| Semen analysis : Pus cell/HPF | >10 | 16 | - | - |
| | 4-10 | 29 | - | - |
| | 2-3 | 55 | 10 | - |

**Continuation of table 1**

| | | | | |
|---|---|---|---|---|
| Sperm count ($10^6$/ml) | Mean ± SD | 28.32 ± 14.60 | 66.5 ± 10.5 | <0.0001* |
| Sperm motility (%) | Mean ± SD | 40.1 ± 13.8 | 64.8 ±7.85 | 0.0045*0.03 |
| Normal morphology (%) | Mean ± SD | 55.92 ± 5.27 | 83.5 ± 5.25 | 8* |
| Total T3 (ng/ml) (0.45 – 1.95) | Mean ± SD | 1.29 ± 0.54 | 1.15 ± 0.55 | 0.25 |
| Total T4 (µg/dl) (4.2 – 9.8) | Mean ± SD | 8.42 ± 1.82* | 7.2 ± 3.2 | 0.02* |
| TSH (µIU/ml) (0.40 – 5.55) | Mean ± SD | 1.98 ± 1.3 | 2.11 ± 1.2 | 0.14 |

*p<0.05

**Table 2** Thyroid status of infertile men

| | Euthyroid (n=69) | Subclinical hypothyroidism (n=31) | P value |
|---|---|---|---|
| **Clinical parameters** | | | |
| Suspected hypothyroidism | 19 | 21 | - |
| Obstructive sleep apnoea | 8 | 4 | - |
| Bipolar affective disorders | 6 | 6 | - |
| Headache | 3 | 1 | - |
| Constipation | 6 | 5 | - |
| Speech problems | 1 | 2 | - |
| **Semen parameters** | | | |
| Oligospermia | 8% | 9% | 0.40 |
| Asthenozoospermia | 7% | 6% | |
| Oligo-asthenoteratospermia | 24% | 20% | |
| Total motile sperm count | 27.5 ± 19.7 x $10^6$ | 26.8 ± 18.4 x $10^6$ | 0.10 |
| Semen volume, mL | 2.9 [1.0–3.2] | 2.6 [1.2–3.6] | 0.45 |
| Sperm motility, % | 42.5 [32.9–37.5] | 37.2 [30.3–36.1] | 0.20 |
| Sperm morphology, % | 47.5 [40.2–60.5] | 30.5 [28.5–55.5] | 0.12 |
| Thyroid profile | 1.38 ± 0.44 | 1.45 ± 0.65 | 0.25 |
| Total T3(ng/ml) | 9.2 ± 1.90 | 8.66 ± 1.7 | 0.02* |
| Total T4 (µg/dl) | | | |
| TSH (µIU/ml) | 1.89 ± 1.1 | 2.32 ± 1.5 | 0.043* |

**Table 3** Comparison of thyroid profile, and risk factors in group I and group II subjects

| | | Group I | | | Group II |
|---|---|---|---|---|---|
| | | Oligozoospermia (n=32) | Asthenozoospermia (n=48) | Oligo-asthenozoospermia (n=20) | |
| Total T3( ng/ml) | Mean ± SD | 1.15 ± 0.4 | 1.29 ± 0.59 | 1.25 ± 0.32 | 1.2 ± 0.40 |
| Total T4 (µg/dl) | Mean ± SD | 7.35 ± 1.42 | 9.15 ± 1.85* | 7.85 ± 1.65 | 7.2 ± 2.15 |
| TSH (µIU/ml) | Mean ± SD | 1.69 ± 0.55 | 2.12 ± 1.45 | 1.88 ± 0.40 | 2.1 ± 1.20 |
| BMI- | Normal | 19 | 22 | 11 | 71 |
| | Overweight | 8 | 14 | 7 | 21 |
| | Obese | 5 | 12 | 2 | 8 |
| Varicocele | | 7 | 14 | 6 | 0 |
| Alcoholic | Mild | 5 | 4 | 4 | 7 |
| | Moderate | 3 | 2 | 2 | 2 |
| | Heavy | 2 | 1 | 1 | 2 |
| Smoker | Light Smokers | 6 | 4 | 4 | 8 |
| | Moderate Smokers | 4 | 2 | 2 | 3 |
| | Heavy Smokers | 2 | 2 | 2 | 2 |

*Mild alcohol: consuming 40g or less; Moderate alcohol: consuming 40-80g; and Heavy alcohol: consuming more than 80g per day. Light Smokers: 01-10 cigarettes/day; Moderate Smokers: 11-20 cigarettes/day; and Heavy Smokers: >21 cigarettes/day.*

Total 30% patients were having smoking habit in the infertile male group. 16 out of 30 smoker had >50% motility while 14 had a motility of <5%. Also 20 patients of the smoking group had <10% of morphologically normal sperm. Oligozoospermia was present in as high in the alcoholics than in the nonalcoholic cases. All three abnormalities like oligo-asthenozoospermia, asthenozoospermia, and oligozoospermia were seen in 32 patients of the alcoholic group.

10 out of 19 obese patients had sperm motility <5%. Totally, 27 patients were found varicocele, among them 14 had asthenozoospermia, 7 had oligozoospermia, and 6 had oligo-asthenozoospermia.

**Relative operating curve (ROC) analysis**

We evaluated diagnostic accuracies of investigative parameters using ROC curve analysis to predict the accurate value for male infertility occurrence (Table 4). ROC analysis between the group I vs the group II showed a significant increase in sperm count in the group I than the group II, however increase in sperm count was not linear (Figure 1. a). Comparative ROC analysis showed a linear response for cases with >28.32 sperm count vs <28.32 million/ml of Sperm count (Figure 1. b.). ROC analysis did not show a linear response for sperm motility in cases with >40.1, sperm motility vs <40.1% (Figure 1. d). ROC analysis did not show a significant change in normal morphology in the group I compared to the group II (Figure 1.e). Comparative ROC analysis did not show a linear response for normal morphology of >55.92 in the group I, and <55.92 in the group II (Figure 1.f.).

ROC analysis did not show a significant change in total T3 levels in the group I compared to the group II (Figure 2.a.). Comparative ROC analysis did not show a linear response for Total T3 levels in cases with >1.29 Total T3 vs <1.29 Total T3 levels (Figure 2.b). ROC analysis did not show a significant change in total T4 levels in the group I compared to the group II (Figure 2c.). Comparative ROC analysis did not show a linear response for total T4 levels in cases with >8.42 Total T4 vs <8.42 Total T4 levels (Figure 2.d.). ROC analysis did not show a significant change in TSH levels in the group I compared to the group II (Figure 2.e.). Comparative ROC analysis did not show linear response for TSH levels in cases with >1.98 TSH vs <1.98 TSH levels (Figure 2.f.).

**Correlation analysis:**

Table 5 shows the Pearson correlation analysis of blood thyroid profile with seminal parameters. There were inverse correlations of thyroid profile with sperm count, sperm motility, and normal sperm morphology. There were significant ($p<0.01$) inverse correlations of serum TSH levels with sperm volume ($r= -0.12$, $p= 0.02$), sperm motility ($r= -0.26$, $p= 0.02$), and sperm morphology($r= -0.304$, $p= 0.02$). There was a significant correlation seen between Serum T4 levels and sperm count ($r= -0.278$, $p= 0.02$), and serum T4 levels and sperm motility ($r= -0.249$, $p= 0.032$).

**Table 4** ROC analysis showing Area under the ROC Curve (AUC), sensitivity and specificity of different parameters

| Parameters | AUC | Sensitivity | Specificity |
|---|---|---|---|
| **Group I vs group II** | | | |
| Sperm count | 0.7860 | 87.50 | 50.80 |
| Total T3 | 0.5315 | 50.46 | 52.31 |
| Normal morphology | 0.6390 | 72.36 | 42.63 |
| Sperm motility | 0.7110 | 69.85 | 50 |
| Total T3 | 0.5030 | 46.91 | 54.23 |
| TSH | 0.5740 | 50 | 57.78 |
| Cases with Sperm motility < 40.1 vs Sperm motility > 40.1 | | | |
| Sperm count | 1.000 | 93.75 | 68.95 |
| Total T3 | 0.5882 | 57.91 | 50.09 |
| Normal morphology | 0.5579 | 48.35 | 60.06 |
| Sperm motility | 0.5671 | 57.91 | 48.03 |
| Total T3 | 0.6278 | 58.54 | 51.96 |
| TSH | 0.5570 | 62.17 | 44.27 |

**Table 5** Pearson correlation of Total T3, T4, and TSH levels with semen parameters in infertile men

| | Total T3 (ng/ml) | | Total T4 (µg/dl) | | TSH (µIU/ml) | |
|---|---|---|---|---|---|---|
| | R value | P value | R value | P value | R value | P value |
| Semen volume (ml) | -0.147 | 0.22 | -0.095 | 0.430 | -0.12 | 0.02 |
| Sperm count ($\times 10^6$ cells/ml) | -0.150 | 0.32 | -0.278 | 0.020 | -0.26 | 0.22 |
| Sperm motility (%) | -0.091 | 0.42 | -0.249 | 0.032 | -0.26 | 0.02 |
| Morphology (%) | -0.079 | 0.45 | 0.160 | 0.130 | -0.304 | 0.02 |

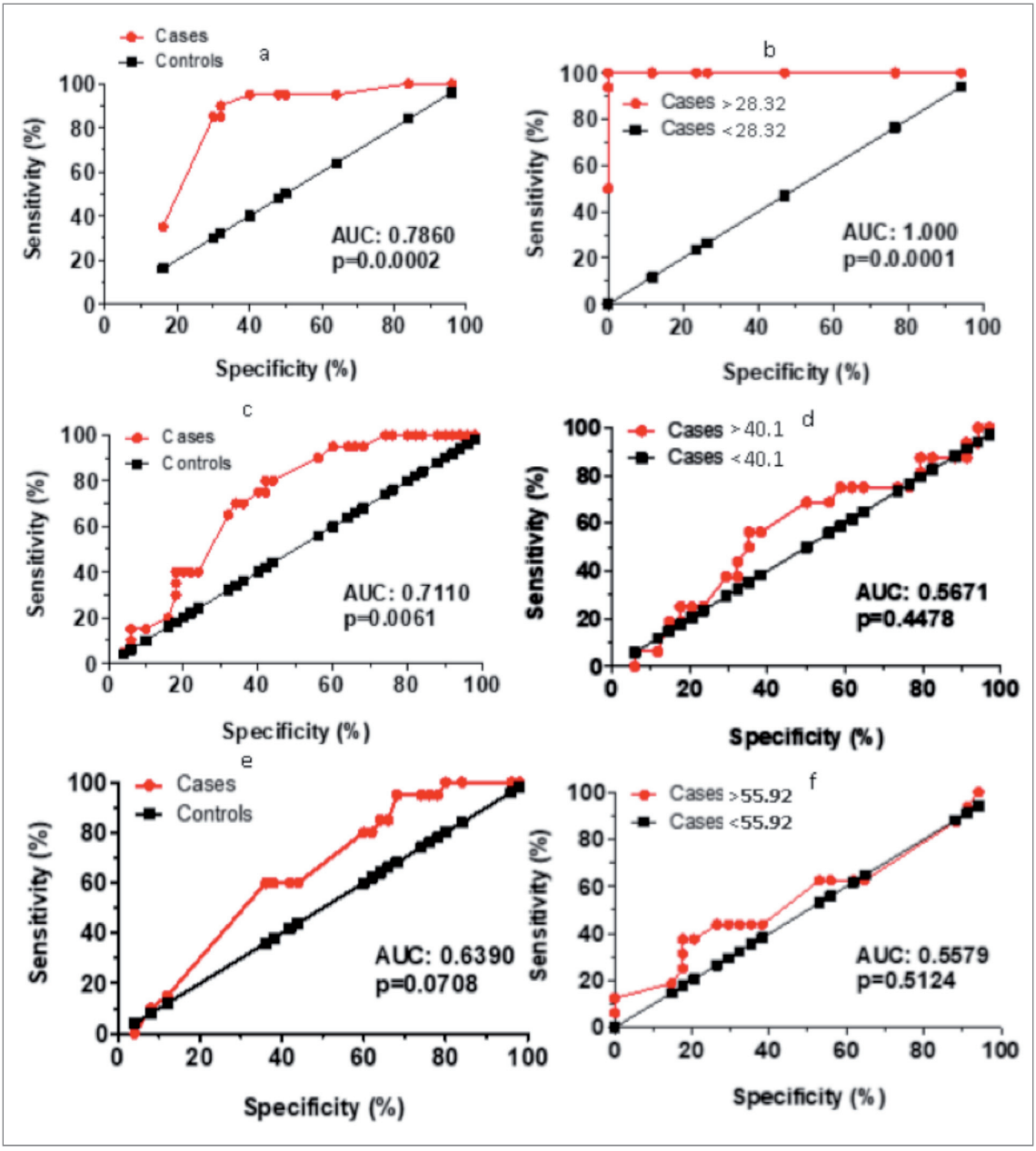


**Fig. 1** a. ROC of sperm count between the group I and the group II. b. ROC of sperm count levels between the cases with sperm count <28.32 to >28.32. c. ROC of sperm motility levels between the group I and the group II. d. ROC of sperm motility levels between the cases with sperm motility value >40.1, and <40.1. e. ROC of normal morphology levels between the group I and the group II. f. ROC of Normal morphology between the cases with normal morphology value >55.92 and <55.92.

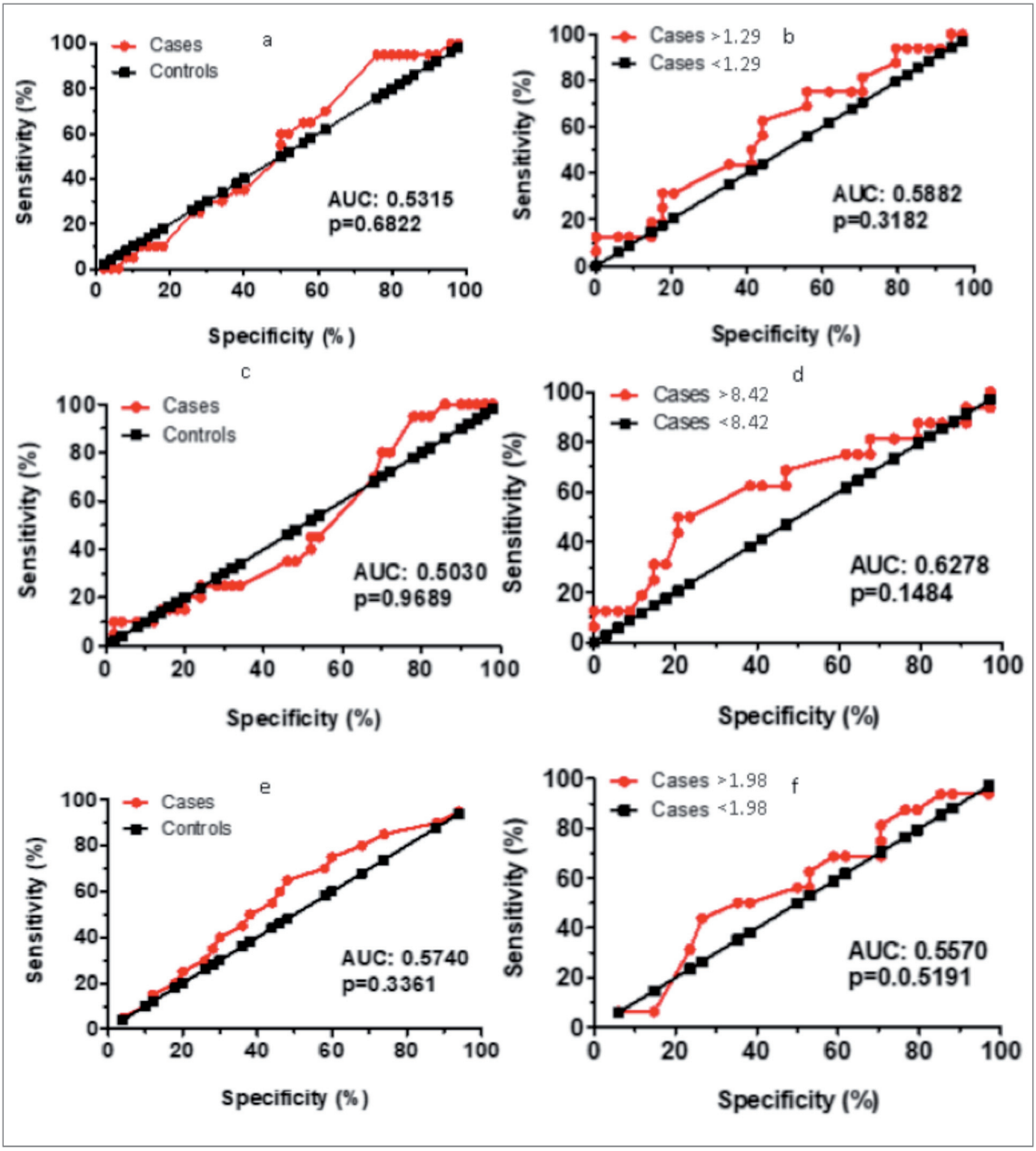


**Fig. 2** a. ROC of total T3 levels between the group I and the group II. b. ROC of total T3 levels between the cases with total T3 value >1.29 and <1.29. c. ROC of total T4 levels between the group I and the group II. d. ROC of total T4 levels between the cases with total T4 value >8.42 and <8.42 .e. ROC of TSH between the group I and the group II. f. ROC of TSH between the cases with TSH value >1.98 and <1.98.

## DISCUSSION

Although several causes of male infertility have been identified, the exact aetiology and pathogenesis of approximately half of all male infertility cases remains unknown.

Oligozoospermia was found in 32 (32%, 95% confidence interval [CI], 27.5% - 35.5%), asthenozoospermia in 48 (48%, 95% confidence interval [CI], 41.5%- 50.5%), and oligo-asthenozoospermia in 20 men in our study of 100 abnormal subjects (20%, 95% confidence interval [CI], 16.85% - 22.8%). Sharma et al., were also observed the similar results (21).

In the current study, it was observed that the majority of subjects were from urban areas, possibly due to pollution or lifestyle-related factors such as stress and other factors that may affects male fertility. In this study, the age range for presentation was 24 to 39 years, with a mean age of 32.4 ± 4.5 years.

In the current study, 35% had 2 to 3 years of active married life, and 33% had 4 - 5 years of active married life, with a mean of 5.1years. After 6 years of active married life, this was reduced to 5% in patients with 2 years of active married life. Hence, the shorter the duration of male infertility, had the better the prognosis.

It was observed that 16 of 30 smokers had a motility of more than 50%, while 14 had a motility of less than 5%. Sperm motility was less than 5% in 8 percent of light smokers, 4% of moderate smokers, and 4 percent of heavy smokers. This study was comparable to that of Zhang et al., (22) and Tandel and Patel (23).

Zukerman et al., also reported that Oligo-asthenozoospermia, asthenozoospermia, and oligo-zoospermia in 32% of alcoholics (24). Villalta`s study showed that Oligo-asthenozoospermia, asthenozoospermia, and oligozoospermia in 15% of smokers and 72% in non-alcoholics (25).

In our study, 10 of 19 obese patients had 5% sperm motility. While 4 out of 29 overweight patients and 4 out of 52 normal weight patients had 50% sperm motility, normal weight patients had 50% sperm motility. Korte et al., observed that men with a high BMI (>25) have a low number of normal-motile sperm cells (26).

Infection of the male reproductive tract is represented by the presence of >4 pus cells in the sperm. A pus cell count of 4-10 pus cells/HPF indicated a moderate infection in 29% of patients, and >10 pus cells/HPF indicated a severe infection in 16% of patients in our study. According to Saxena, 20% of cases had severe infections (27).

In our study, 27% of the cases had varicocele, 14% had asthenozoospermia, 7% had oligospermia, and 6% had oligo-asthenozoospermia. Ali study showed 36% asthenozoo-spermia, 25% oligospermia, and 21% teratozoospermia (28, 29).

Thyroid hormone levels have been shown to correlate positively with serum testosterone levels. This condition is associated to a number of male reproductive disorders, including hypoactive sexual behaviour, erectile dysfunction, delayed ejaculation, and poor sperm quality (30). Various studies have described an association between the hypothyroidism and male infertility, but it is unclear that hypothyroidism may directly affects the sperm quality (31-34).

In our current study, 31% of the subjects had a subclinical hypothyroidism. Subclinical thyroid dysfunction was associated with an altered sperm count but not sperm motility or morphology. This finding is consistent with the findings of other studies (35).

There were inverse correlations of thyroid profile with sperm count, sperm motility, and normal sperm morphology. There were significant ($p < 0.01$) inverse correlations of serum TSH levels with sperm volume ($r= -0.12$, $p= 0.02$), sperm motility ($r= -0.26$, $p= 0.02$), and sperm morphology($r= -0.304$, $p= 0.02$). Similarly, serum T4 levels were significantly ($p<0.01$) correlated with sperm count ($r= -0.278$, $p= 0.02$), and sperm motility ($r= -0.249$, $p= 0.032$).

Both hyperthyroidism and hypothyroidism affects the testicular function and neuroendocrine regulation of reproductive functions via the hypothalamic-pituitary-thyroid (HPT) and hypothalamic-pituitary-gonadal (HPG) axis crosstalk. TSH is critical in the regulation of the HPT axis. The HPG axis normally regulates testicular function. Thyroid hormones work biologically by binding to two nuclear thyroid hormone receptors (TRs): TR and THR. Thyroid hormones are also important in the initiation of Leydig cell differentiation before puberty (36).

T3 binds to TRs on postpubertal Sertoli cells, inhibiting the synthesis of aromatase and androgen-binding protein, as well as modulating testosterone conversion to 17-estradiol and testosterone concentration in the seminiferous tubules. Thyroid hormones promote testosterone steroidogenesis in adult Leydig cells by activating the steroidogenic acute regulatory (StAR) protein, which is involved in cholesterol transport into the mitochondria (31).

Many conditions may influence the sperm quality of males. Oxidative stress is well-known to be harmful to sperm quality, primarily by causing DNA damage in sperm cells. Thyroid hormone physiological ranges regulate metabolism, including cell oxygen consumption, and both hyperthyroidism and hypothyroidism are associated with oxidative damage.

A low sperm volume was reported in a small series of subclinical hypothyroidism (n =31); in our study, no subjects were overtly hypothyroid/hyperthyroid, though a few had lower TSH levels (32, 33).

In our study, the mean T3, T4, and TSH levels in infertile male subjects were 1.29 ± 0.54 ng/ml, 8.42 ± 1.82 g/dl, and 1.98 ±1.3 IU/ml, respectively. Within the normal range of TSH values, the relationship of TSH with the sperm parameters was more pronounced in our study. In our study, 31% of the subjects had subclinical hypothyroidism and lower sperm counts. In other studies, 24-25% of hypothyroid subjects had lower sperm counts, with no significant difference in sperm volume (34-38).

In the current study, total T3 of oligozoospermic men with a mean of 115 ± 0.4 ng/ml, total T4 with a mean of 7.35 ± 1.42 µg/dl, and TSH with a mean of 1.69 ± 0.55 IU/ml, whereas in asthenozoospermic men total T3 with mean of 1.29 ± 0.59 ng/ml, total T4 with the mean of 9.15 ± 1.85 µg/dl, and TSH ranged with mean of 2.12 ± 1.45 IU/ml.

Clyde et al. (39) studied three young thyrotoxic males and observed that two had marked oligozoospermia with a decreased motility and the third had borderline low sperm counts with a decreased motility. Kidd et al. (40) studied Grave's disease patients and observed that four of the five had total sperm counts less than 40 million/ml and only one had sperm density less than 24 million/ml.

Furthermore, total T4 was found to be significantly higher in men with asthenozoospermia, but no relationship between T3 and/or TSH and oligozoospermia, asthenozoospermia, or oligoasthenozoospermia was found, which is consistent with Kumar et al. (19).

Our findings are consistent with the findings of Lotti et al. (18), who observed that euthyroid subjects (n=145) had lower semen volumes compared to those with subclinical hyperthyroidism (n=6) and higher than those with subclinical hypothyroidism (n=12); however, no relationship between the semen parameters and TSH was found. Thyroid function abnormalities appear to have the most significant effect on sperm parameters, namely impaired motility and/or morphology (41).

Hence, the most noticeable effect of thyroid function abnormalities on sperm parameters was impaired motility and/or morphology. Based on our findings, we concluded that the relationship between TSH and sperm volume/count was stronger.

## CONCLUSION

Smoking, obesity, and infection all reduce fertility by lowering sperm count, motility, and changing the morphology of sperm. We observed a significant relationship between TSH and sperm volume and motility. These findings suggest that normal thyroid function is a component of good sperm quality.

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**Conflict of interests:** Authors declare that there is no conflict of interest

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# EVALUATION OF ACUTE PANCREATITIS BASED ON BISAP SCORING SYSTEM: A COHORT STUDY OF 50 CASES

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## Abstract

Background/Aim: Acute pancreatitis is encountered in both medical and surgical specialty. Assessment of severity and grading is done using radiological investigations mostly like ultrasonography or CECT. We present a study to assess the severity of Acute Pancreatitis based on Bedside Index for Severity in Acute Pancreatitis (BISAP) scoring system.

Material & Methods: The study was conducted on 50 patients presenting with acute pancreatitis who were included as per inclusion criteria and a detailed history, clinical examination and blood investigation performed. Data like serum amylase, serum lipase, serum calcium, blood urea nitrogen (BUN), pleural effusion and systemic inflammatory response syndrome (SIRS) was collected from the patients. Based on data collected in 24 hours of hospitalization, BISAP score was calculated.

Results: Results showed that no significant temperature rise, pancreatic necrosis, SIRS or impaired mental status in patients with BISAP severity of <=3. However, patients having BISAP score >3, factors like BUN, age, pleural effusion, and organ failure show significant correlation. Also on comparative analysis of patients showed that the hospital stay, respiratory rate, pulse and laboratory markers (blood urea, serum creatinine, serum amylase, serum lipase) were significantly higher in patients with BISAP score ≥3.

Conclusion: BISAP score is an easy, quick and bedside method to assess the severity of acute pancreatitis and predict its mortality. It is easy bedside procedure that can be done in every setup.

**Key words:** Acute Pancreatitis, Multi organ failure, Mortality

## INTRODUCTION

Acute pancreatitis (AP) is an abdominal condition caused by pancreatic inflammation and patients presents with pain in abdomen and there is rise in pancreatic enzyme levels in the blood or urine.[1] It has always been a great challenge for the treating surgeon in their routine surgical practice as AP is common entity encountered in emergencies. Although most of the cases of AP resolves spontaneously, but 30% mortality is seen in severe acute pancreatitis.[2] The etiologies and natural histories are complex and variable that makes it difficult to detect high risk patients at early stage. Incidence of pancreatitis is different in different geographical locations but common etiologies are alcohol, gallstones, metabolic factors, drugs and idiopathic.[3]

Identification of severe AP after admission is of great clinical significance as it will help in triage and starting aggressive early treatment. Many guiding principles have been followed over the years that have evolved and also borne out of certain studies. The strategies that

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have been used to assess the severity of acute pancreatitis are Ranson's criteria, APACHE II (Acute Physiology and Chronic Health Evaluation II) scoring, CTSI (Computed Tomography Severity Index), Glasgow scoring systems. Although none is recognized as standard criteria, each one has its advantages and disadvantages.[4-6]

Wu et al in 2008 developed a quick and accurate method for recognition of high risk patients which is called BISAP (Bedside Index for Severity in Acute Pancreatitis) scoring system. This system provides an accurate, easy, quick, simple and reproducible description of disease severity.[7]

Since the data for BISAP score is collected within 24 hours of hospitalization, patients can be stratified early according to those who are at high risk for mortality and organ failure. As BISAP scoring is quick and bedside method, patients can be assessed in their early course and thus it can help improving future management strategies in acute pancreatitis.[8]

Individual components of BISAP Score include:

1) Blood urea nitrogen> 25mg/dl,
2) Mental status of patient (Glasgow coma scale score),
3) Systemic inflammatory response syndrome (SIRS)

Presence of more than 2 of following criteria (Score of > 3 will indicate early organ failure or pancreatic necrosis):

- Pulse >90 bpm,
- Respiration >20/min or PaCO2 <32 mm Hg,
- Temperature ≥38 degree celsius or <36 degree celsius,
- WBC >12000 or < 4000 cells/$mm^3$ or >10% immature neutrophils,

4) Age >60 years and
5) Pleural effusion (on CT scan or chest x- ray or USG).

Each point on BISAP score is worth 1 point. The risk of mortality increases with increase in points. BISAP score is a reliable, quick and uncomplicated method to assess severity at time of admission.[9]

Thus, in order to improve the survival accurate prediction of severity is important. To predict the prognosis and seriousness of acute pancreatitis, multiple assessment criteria are used to guide patient assessment and management. However, nothing has been shown to outperform good clinical judgment in clinical settings. Ideal predicting criteria are those which are simple, non-invasive, accurate and quantitative and assessment tests are readily available.

## MATERIALS AND METHODS

This prospective cohort study was carried out between December 2020 and November 2021 at our institution after approval from the Institutional Ethical Committee. Informed consents of patients were taken. The study was conducted on 50 patients. The case of acute pancreatitis was diagnosed based on the presence of any two of the following features:

- Abdominal pain with features of acute pancreatitis (acute onset of a persistent, severe, epigastric pain often radiating to the back),
- Raised pancreatic enzymes (Serum amylase and/or lipase at least three times greater than the upper limit of normal value),
- Diagnosis of acute pancreatitis on transabdominal ultrasonography or CECT.

Inclusion criteria include:

Based on mentioned criteria all diagnosed cases of acute pancreatitis will be included.

Exclusion criteria include:

- Refusal to participate,
- Organ failure (At the time of admission and/or within 24 hours of presentation),
- Presentation >48 hours of onset of pain and
- Hyperamylasemia of other causes
- Carcinoma pancreas

A detailed history like age, gender, etiologies for pancreatitis, past history of alcohol intake was collected. Clinical examination and blood investigation were performed. Presenting symptoms like fever, abdominal pain, abdominal distension, nausea and vomiting were noted for each patient along with

1) Physical examination
2) Clinical evaluation of patient
3) Appropriate diagnostic procedures.

Data like serum amylase, serum lipase, serum calcium levels, BUN (blood urea nitrogen), pleural effusion and features of SIRS (systemic inflammatory response syndrome) were collected in all the patients. Based on data obtained within 24hr of hospitalization, BISAP score was calculated.

Statistical analysis:

Statistical analysis was done on Microsoft excel sheet (2010) and SPSS software version 21. Chi square test was used for finding association between variables. P value of <.05 was considered significant.

## RESULTS

Table 1 shows the baseline characteristics of the patients. Figure 1 shows that most common etiological factor was idiopathic (32%, n=16) followed by gall stone (28%, n=14) and alcohol (24%, n=12). In 8% (n=4) cases hyper triglyceridemia was the cause. Drug induced and post ERCP contributed 4% (n=2) cases each. In present study 24% (n=12) cases presented with pancreatic necrosis and the mortality rate was 2%. Figure 2 shows that 64% (n=32) cases have BISAP score ≤2 while 36% (n=18) cases have BISAP score ≥3. However there was no significant correlation found between etiology and BISAP score of pancreatitis (Table 2).

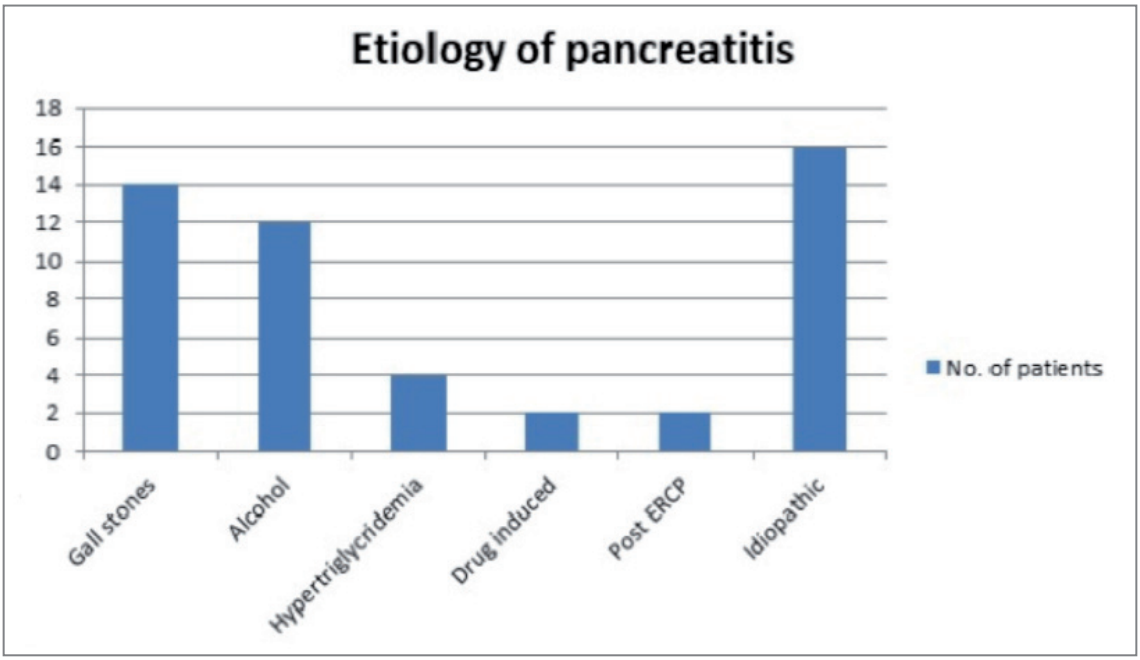


**Fig. 1** Etiology of Pancreatitis

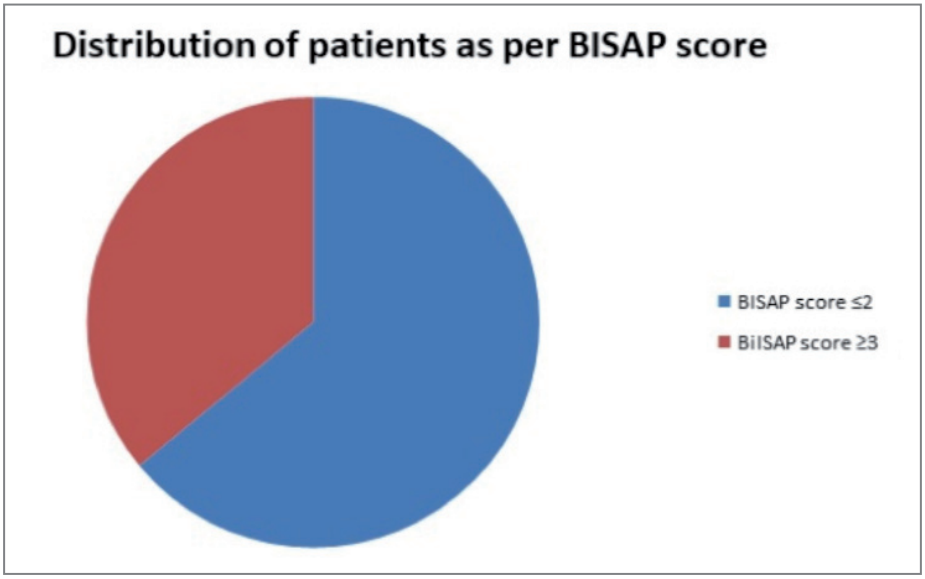


**Fig. 2** Patients with BISAP score of <2 and >3

Statistically significant values were observed for BUN, age, pleural effusion, and organ failure in patients with BISAP score >3 (Table 3).

In table 4 the comparative analysis of patients having BISAP score ≤2 (n=32) and ≥3 (n=18) is shown It was observed that significantly higher values for hospital stay, respiratory rate, pulse, blood urea, serum creatinine, serum amylase, and serum lipase is present in patients with BISAP score ≥3.

**Table 1** Baseline characteristics of the patients

| Parameters(n=50) | Mean/n | Std. Deviation/% |
|---|---|---|
| AGE (YEARS) | 41.92 | 15.018 |
| SEX (male ) | 25 | 50% |
| HOSPITAL STAY(DAYS) | 11.34 | 3.497 |
| TEMPERATURE (Degree C) | 38.34 | 0.626 |
| RESPIRATORY RATE (breaths per minute) | 17.48 | 1.182 |
| PULSE (beats per minute) | 94.14 | 2.483 |
| TLC/$mm^3$ | 12942.06 | 1723.282 |
| NEUTROPHILS | 78.16 | 4.287 |
| LYMPHOCYTES | 47.54 | 9.072 |
| BASO | 0.04 | 0.198 |
| MONOCYTES | 2.58 | 0.785 |

**Continuation of table 1**

| | | |
|---|---|---|
| EOSINOPHIL | 5.06 | 1.812 |
| BLOOD UREA (mg/dL) | 88.72 | 88.005 |
| SERUM CREATINE mg/dL | 1.588 | 0.9323 |
| SERUM AMYLASE (U/L) | 359.92 | 111.241 |
| SERUM LIPASE(U/L) | 698.48 | 195.474 |
| SERUM CALCIUM (mg/dL) | 8.69 | 0.7726 |

**Table 2** RELATIONSHIP OF BISAP SCORE SEVERITY WITH ETIOLOGY

| **BISAP** | **≤2** | | **≥3** | | **Total** | | **Chi square value** |
|---|---|---|---|---|---|---|---|
| **Etiology** | n | % | n | % | n | % | 647 |
| Hypertriglyceridemia | 3 | 9.40 | 1 | 5.60 | 4 | 8.00 | |
| Gall stones | 7 | 21.90 | 7 | 38.90 | 14 | 28.00 | |
| Idiopathic | 10 | 31.30 | 6 | 33.30 | 16 | 32.00 | |
| Alcoholic | 9 | 28.10 | 3 | 16.70 | 12 | 24.00 | |
| Drug induced | 2 | 6.30 | 0 | 0.00 | 2 | 4.00 | |
| Post ERCP | 1 | 3.10 | 1 | 5.60 | 2 | 4.00 | |
| Total | 32 | 100 | 18 | 100 | 50 | 100 | |

**Table 3** Relationship of BISAP score severity with various parameters

| BISAP | ≤2 | | ≥3 | | Total | | Chi Square |
|---|---|---|---|---|---|---|---|
| | N | % | N | % | N | % | P-Value |
| Temperature (≥39) | 12 | 37.50% | 9 | 50.00% | 21 | 42.00% | .390 |
| Pancreatic Necrosis | 6 | 18.75% | 6 | 33.30% | 12 | 24.50% | .273 |
| BUN(>25) | 2 | 6.30% | 16 | 88.90% | 18 | 36.00% | .001 |
| Age(>60) | 0 | 0.00% | 8 | 44.40% | 8 | 16.00% | <.001 |
| Pleural Effusion | 17 | 53.10% | 17 | 94.40% | 34 | 68.00% | .003 |
| Impaired Mental Status | 0 | 0 | 0 | 0 | 0 | 0 | - |
| SIRS | 32 | 100.00% | 18 | 100.00% | 50 | 100.00% | .515 |
| Organ Complication | | | | | | | |
| No Complication | 32 | 100.00% | 0 | 0.00% | 32 | 64.00% | <.001 |
| ARDS | 0 | 0.00% | 2 | 11.10% | 2 | 4.00% | |
| Cardiac | 0 | 0.00% | 1 | 5.60% | 1 | 2.00% | |
| Mods | 0 | 0.00% | 2 | 11.10% | 2 | 4.00% | |
| Renal | 0 | 0.00% | 13 | 72.20% | 13 | 26.00% | |
| Total | 32 | 100.00% | 18 | 100.00% | 50 | 100.00% | |

**Table 4** Comparison of mean in according to severity of BISAP score with different parameters

| BISAP | ≤2 (n=32) | | ≥3(n=18) | | t-value | p-Value |
|---|---|---|---|---|---|---|
| | Mean | Std. Deviation | Mean | Std. Deviation | | |
| HOSPITAL STAY(DAYS) | 10.28 | 2.738 | 13.22 | 3.96 | -3.094 | 0.003 |
| TEMPERATURE (Degree C) | 38.28 | 0.63 | 38.44 | 0.61 | -0.88 | 0.38 |
| RESPIRATORY RATE (breaths per minute) | 17.19 | 1.12 | 18 | 1.13 | -2.44 | 0.01 |
| PULSE (beats per minute) | 93.5 | 2.12 | 95.28 | 2.71 | -2.56 | 0.01 |
| TLC/$mm^3$ | 12652.97 | 1873.79 | 13456 | 1311.53 | -1.60 | 0.11 |
| NEUTROPHILS | 77.63 | 4.61 | 79.11 | 3.54 | -1.18 | 0.24 |
| LYMPHOCYTES | 46.91 | 8.49 | 48.67 | 10.16 | -0.65 | 0.51 |
| BASO | 0 | 0 | 0.11 | 0.32 | -1.9 | 0.056 |
| MONOCYTES | 2.47 | 0.76 | 2.78 | 0.80 | -1.34 | 0.18 |
| EOSINOPHIL | 4.84 | 1.81 | 5.44 | 1.79 | -1.12 | 0.26 |
| BLOOD UREA (mg/dL) | 39.72 | 5.68 | 175.83 | 98.53 | -7.85 | <0.001 |
| SERUM CREATININE mg/dL | 1.022 | 0.25 | 2.594 | 0.843 | -9.84 | <0.001 |
| SERUM AMYLASE (U/L) | 329.63 | 63.20 | 413.78 | 153.57 | -2.73 | 0.009 |
| SERUM LIPASE(U/L) | 624.16 | 171.58 | 830.61 | 166.02 | -4.13 | <0.001 |
| SERUM CALCIUM (mg/dL) | 8.6 | 0.87 | 8.85 | 0.54 | -1.10 | 0.27 |

**Table 5** Comparison of BISAP score between different studies

| BISAP SCORE | SCORE≤2 | SCORE ≥3 |
|---|---|---|
| Present study (2021) | 64% | 36% |
| Chittipotula et al(2020)[8] | 64% | 36% |
| Hagjer et al(2017)[9] | 80% | 20% |
| Chandrashekhar et al[11] | 82% | 18% |
| Venkatapuram MR et al. (2018)[12] | 64% | 36% |
| Veena P et al (2020)[14] | 64% | 36% |
| Katta et al (2016)[15] | 74.51% | 25.49% |

## DISCUSSION

The study was conducted to assess efficacy of BISAP score in correctly evaluating severity of acute pancreatitis at time of admission/presentation.

Maximum patients were of age group <40 years (54%) and the mean age was 41.92±15.01 years. Range of age varied from 38.62-46.31 years. The results are comparable to previous studies.[9-12] Equal number of male and female was observed with male: female ratio of 1:1. However male predominance is seen in most studies but ratio varies from 2:3 to 9:1.[11-15]

In present study the commonest cause was idiopathic (32%) followed by gallstone disease (28%). Similar results were shown by Kanase et al, where 31% patients had idiopathic cause while 28% patients had gall stone disease.[16] In study conducted by Singh et al, 27% patients had gall bladder stones as etiological factor which was most common cause followed by alcohol (21.4%) and idiopathic (15.1%).[17] However Venkatapuram et al, reported alcohol as most common etiological factor (80%) followed by gall bladder stones (8%).[12] Gall bladder stones and alcohol are common etiological factors for acute pancreatitis. However present study showed idiopathic as most common cause.

In 76% (38) cases pancreatic necrosis was absent while in 24% (12) cases it was present. The incidence of pancreatic necrosis was seen between 14%–35% in various studies.[8,9,12,14,17] Similar findings were seen in present study.

In our study 98% patients were discharged in satisfactory condition while mortality rate was 2%(n=1). However, the mortality rate varies from 3.5%-16%.[8,9,12,15,17] Small sample size could be the reason for low mortality rate in present study.

In present study it was seen that 64% (n=32) cases have BISAP score ≤2 while 36% (n=18) cases have BISAP score ≥3. Mostly in all the studies majority of the patients have mild pancreatitis, which was also observed in present study. BISAP score of present study is compared with other studies (Table 5).

It was observed that there is significant association between age group and BISAP score (p=.002). Also patients with BISAP score ≤2 has age ≤60 years while among the cases with BISAP score ≥3, 55.60% have age ≤60 years and 44.40% have age >60. This association was statistically significant.

There is no significant association observed between pancreatic necrosis and BISAP severity score in both the groups. There is significant association between organs complications and BISAP score as all complications were seen in patients with BISAP score ≥3. Singh et al, also found significant increase in organ failure and pancreatic necrosis with increase in BISAP score.[17] Senapati et al found the same results with significant association between pancreatic necrosis and BISAP score. [18] Khanna et al also observed that

patients with ≥3 BISAP score have more pancreatic necrosis and organ failure compared to other. [19]

Senapati et al, also observed that organ failure and pancreatic necrosis is higher among patients with BISAP scores of 3 as compared to those with score od <3.[18] Although persistent organ failure and pancreatic necrosis are two important predictors of outcome in acute pancreatitis but presence of both factors have greater effect than either determinant alone. According to Senapati et al organ failure and infected necrosis are two independent and equivalent determinants of mortality in acute pancreatitis and there is two fold increase in mortality when both are present.[18]

It has been reported that BISAP scores of ≥3 carry a 7.4-fold higher risk of developing organ failure and 12.7-fold higher risk for persistent organ failure. Organ failure is considered as much stronger predictor of mortality than the extent of necrosis.[17] Overall mortality rate reported by Perez et al, was 14% among 99 patients with pancreatic necrosis but it was 50% when there is concomitant presence of organ failure at or during hospitalization.[20] Rau et al, observed a 19-fold increased risk of mortality in patients having sterile necrosis and multisystem (>2) organ failure, treated either operatively or conservatively.[21] Persistence of organ failure is a major determinant of mortality in acute pancreatitis than organ failure.[22,23]

In 93.80% (n=30) cases with BISAP score ≤2 have BUN≤25 and majority patients with BISAP score ≥3 have BUN >25 i.e. 88.89% (n=16). This association was statistically significant (p<.001). Also, in present study the average BUN for severe pancreatitis was 175.83mg/dl and for mild it was 39.72 mg/dl with highly significant difference between the two. Pre-renal azotemia caused by initial hypovolemia lead to rise in the BUN level at admission in patients with acute pancreatitis. Acute pancreatitis, and impairment of renal function lead to increased protein catabolism that cause negative nitrogen balance which further leads to azotemia.[24,25] Elevated BUN at time of admission is increases the risk of mortality which makes it an independent risk factor in acute pancreatitis.[7,25] Koutroumpakis et al. observed in their study that rise in BUN at 24 hour is better predictor of persistent organ failure and pancreatic necrosis in acute pancreatitis than other laboratory markers.[26] Our results are in accordance with these studies with respect to initial increased BUN level at admission and its association with the development of SAP in acute pancreatitis.

Also, in patients with BISAP score of ≥3, 94.40% patients had pleural effusion while in those with BISAP score of ≤2, 53.10% had pleural effusion. This association was statistically significant (p=.003). The cause of pleural effusion observed during acute pancreatitis could be due to leakage of pancreatic secretions directly into the pleural cavity via the trans diaphragmatic lymphatic channels due to pancreatic duct disruption. Maringhini et al, in their study observed an increased in incidence of pseudocyst formation related to acute pancreatitis when there is associated pleural effusion.[27] Heller et al, also showed association between pleural effusion on chest x-ray and severity as per BISAP score.[28] The present study suggests that pleural effusion is a strong individual predictor of severe acute pancreatitis according to BISAP criteria.

It was observed that hospital stay, respiratory rate, pulse, BUN, serum creatinine and pancreatic enzymes (serum amylase, serum lipase) were significantly more in patients with BISAP score ≥3.

The mean hospital stay for the patients was 11.34±3.4 days. Chandrashekhar et al, in their study found that hospital stay was 7.16 days which was less than the present study.[11] In present study it was seen that with increase in BISAP score the duration of hospital stay increases. Mean hospital stay was 10.28 days for mild acute pancreatitis while it was 13.5 days for severe acute pancreatitis which was longer then the study by Chittipotula et al where mean duration of hospital stay was 4.8 days and 8.3 days for mild acute pancreatitis severe acute pancreatitis respectively.[8] According to a study by Hagjar et al the mean hospital stay in patients with BISAP score of ≤2 was 7.52 ± 3.053 days while it was 10.429 ± 5.872 days for BISAP score ≥3 days.[9]

The study had certain limitations that include small sample size and short duration of study. Moreover being a more clinical and indirect method, many would prefer to opt for CECT early.

But still BISAP score is effective in finding out the severity and predicting mortality in patients with acute pancreatitis. It is a very useful tool to triage patients into mild and severe disease. Being easy to perform and as bedside procedure, it can be done in every setup. In conclusion, the ability of the BISAP score to predict mortality and severity in acute pancreatitis is evaluated. In our opinion, BISAP score can be used to stratify patients at risk of mortality within 24 hours of presentation and it can help to improve clinical care and facilitate enrolment of appropriate patients with acute pancreatitis in future prospective trials. However it is indirect method to assess the severity of pancreatitis but useful in places where facilities of CECT and other diagnostic procedures are absent.

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# SIMULATOR TRAINING IN ANEURYSM CLIPPING

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## Abstract

Due to an increased number of patients with aneurysms treated endovascularly, a resident does not have enough opportunities to come across such a complex procedure as surgical clipping. There have been many types of training methods designed for traditional surgical aneurysm treatment so far. A brief report regarding the helpfulness of vascular simulator AneurysmBox at our Neurosurgical Department is presented.

**Key words:** aneurysms, neurosurgical training, AneurysmBox

Ruptured vascular malformations are considered to be a neurosurgical emergency, and rapid evaluation and management might be live-saving. There are various options in aneurysms treatment including endovascular procedures such as coiling and stenting, and then traditional surgical approach using "open" craniotomy followed by a treatment of aneurysm itself, most often by clipping. Currently, endovascular methods are dominating and surgery is limited to a small number of cases when there is a ruptured aneurysm located on middle cerebral artery, usually associated with an expanding intracerebral hematoma, which requires evacuation during the procedure as well. There is another limitation considering "open" surgery – antiplatelet or anticoagulant therapy, which is, on the other hand, necessary for endovascular treatment.

Aneurysm clipping remains a challenge for residents and attending neurosurgeons as well. Due to a low number of aneurysm clippings in our daily neurosurgical practice as endovascular treatment is increasing, neurosurgical training is considered to be very important. A resident can come across this kind of surgery as an assistant or by operating under a supervision in the advanced phase of residence program (1; 2). Regarding the limited number of surgeries performed, there is a lack of opportunity to get familiar with this complex procedure.

Aiming to gain microsurgical skills in aneurysm clipping, lots of different types of simulation activities have been introduced. Various kinds of static simulators, virtual-reality based simulation techniques, and 3D models are most commonly described in aneurysm clipping training. Each of these methods has its own benefits and drawbacks, in general the most limiting disadvantage is a lack of realistic conditions (3; 4). A recent study by Joseph et al. (5) highlighted functional simulators, being able to display realistic anatomy and physiology with blood flow and pulsatility based on obtained image data by specific patients. For successful neurosurgical training in aneurysm clipping, the ideal simulator should be able to provide a detailed anatomy of the brain and vascular structures,

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and also replicate the possible surgery pitfalls, namely periprocedural aneurysm rupture (6; 7).

We got an opportunity to use an AneurysmBox from UpSurgeon. This 3D model contains a white base with skull, a pre-existing standard pterional craniotomy, surgical tools – loupes with LED light, clips applicator, spatula, bayonet, and 4 clips of various shapes. The model mimics realistic vascular anatomy with 5 aneurysms localized on different cerebral arteries. Our goal was to identify and treat all the aneurysms with a proper clip placement, concurrently evaluating the simulator design. Due to a low number of residents at our neurosurgical department, no questionnaires or further research were performed.

In general, we consider this type of training especially beneficial for residents in the first years of their practice. From our point of view the biggest advantage of the use of AneurysmBox is the vascular anatomy highly resembling realistic conditions (congenital vascular anomalies as atypical branching are not displayed). Having more shapes of clips enables choosing the most suitable one aiming to secure appropriate aneurysm closure (Fig. 1,2).

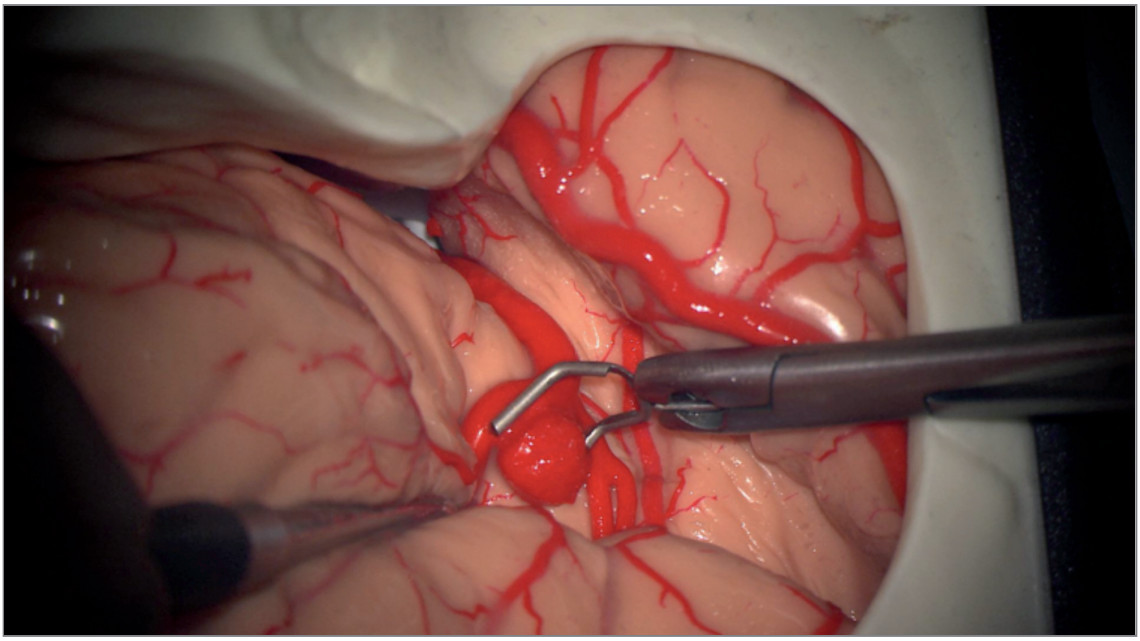

**Fig. 1** Clip placement on aneurysm's neck

Due to various aneurysm locations, a resident is able to train reaching different arterial location and get familiar with surrounding anatomy (Figure 2).

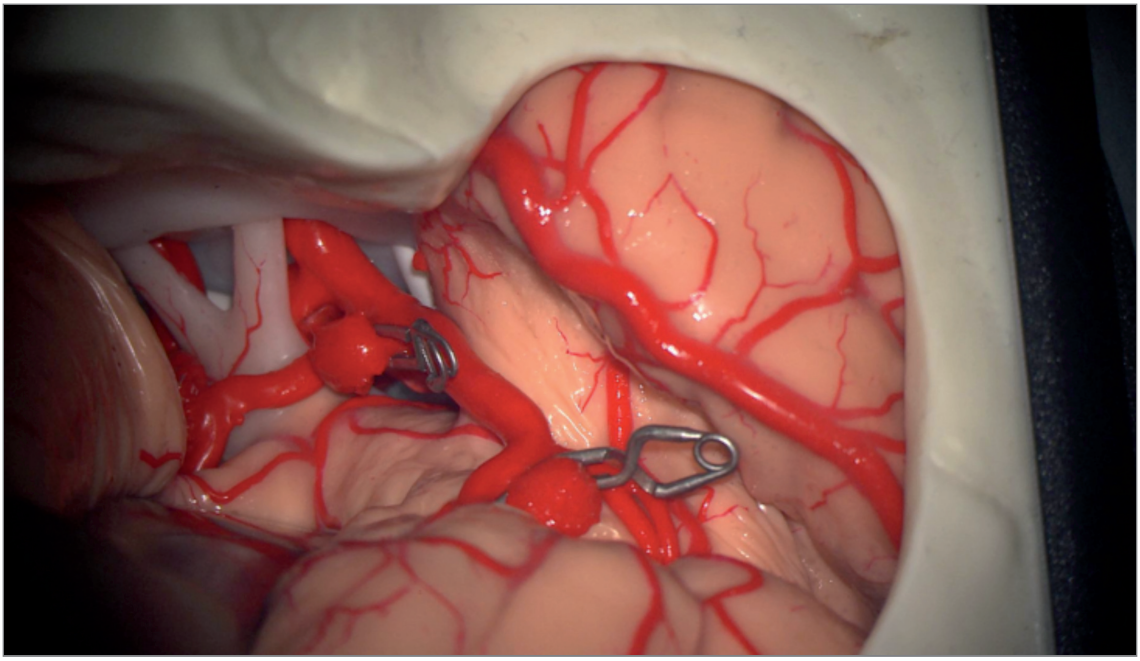

**Fig. 2** Clips placed on middle cerebral artery

On the other hand, the model lacks the properties of potential realistic perioperative complications such as aneurysm rupture or clip malposition resulting in inadequate occlusion. Another limitation could be the material used for the brain model itself, as it does not reflect the softness of tissues and vessels, so inappropriate rough manipulation which would in vivo lead to a vessel damage has basically no impact on the simulator.

To summarize, we would definitely recommend AneurysmBox as a means of aneurysmal clipping training for residents. The biggest benefits appreciated are in getting familiar with surgical approach and vascular anatomy and learning how to manipulate with specific surgical tools.

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