Global Journal of Medical Research: B
Pharma, Drug Discovery, Toxicology and Medicine
Volume 15 Issue 3 Version 1.0 Year 2015
Type: Double Blind Peer Reviewed International Research Journal
Publisher: Global Journals Inc. (USA)
Online ISSN: 2249-4618 & Print ISSN: 0975-5888

Drug-Induced Disparities in Cell Restoration and Debridement: Ethanol-Triggered Nuclear Misreading of the Restitution Cues

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GJMR-B Classification : NLMC Code: QV 738

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I. Introduction

After organ developmental growth is completed, the restitution program remains and determines the preservation and maintenance of the cells structures and their optimal function in the organ. The fidelity of the renewal relies on a fine tuning of the renovation processes with removal of the resected components by the catabolic debridement [1]-[4]. The intricacy of the seemingly simple, orderly, and methodical process resides in a control of the counteractive biosynthetic processes that fuel the restorative effects [1] [2] [5]-[7]. The activities involve the coordinated synthetic means that power renewal of the cellular organelles and cell membrane by genesis of the fragments rebuilding specific organelles, including those responsible for catabolic cleansing of the cell [1]-[3]. Therefore, the opposing in function actions, intimately connected at the stage where specific sets of mRNA undergoing translocation and translation, concomitant with vesicular membrane synthesis, determine intercalation of the protein into proper lipid arrangements and that both elements are compatible with the structure of the organelle undergoing refurbishing [1] [2] [8] [9]-[11]. Distinctly, the restitution necessitates attuned release of the ER-generated transport vesicles destined to maintain and uphold structural and functional aspects of the cell, including structural and digestive features of the restitution program [11]-[14]. Henceforth, the departure from the norm, embodied in alteration of the homeostatic stable equilibrium predetermining quantities of transport vesicles destined to Golgi and/or mitochondria, may be manifested in pathological outcomes exhibited in a variety of medically recognized complications [15]-[19].

In our studies, the experimentally-induced deviation from the homeostatic equilibrium in the production of Golgi-directed transport vesicles was observed while employing ER with the outer leaflet depleted of serine palmitoyltransferase (SPT) activity [2].The elimination of the outer leaflet SPT (OL SPT) curtailed synthesis of endosome/lysosome-directed transport vesicles without noticeable impact on Golgi-directed vesicular transport responsible for the restitution of Golgi, cellular membrane, and apical and/or basolateral secretion [2] [19]-[21]. Thus, likelihood opened that the agents known to impact the ER to Golgi transport may affect assembly of the diverse sets of Golgi-directed vesicles and generate cellular imbalance by suppressing or promoting Golgi restitution, secretion, cell membrane turnover and lysosomal degradation, or fostering cell restoration and accumulation of undigested autophagosomes [20]-[26]. The demarcation of the outer- (OL) and inner (IL) leaflet SPT activity, by tracking the assembly of the vesicles explicitly minted for their specific contribution in the renewal of the organelles responsible for the cell debridement (OL SPT) from those involved in the Golgi and cell membrane restitution (IL SPT), led us to a thought that disturbed balance in membrane replacements may also affect Golgi's constitutional

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turnover and/or repair [2] [24]-[26]. Thus far, the vesicles-driven restitution of Golgi organelle was not viewed as the separate process that determines the physical fitness of the site that transforms ER-derived vesicles into cell organelles-specific entities. With these features in mind, ethanol, the toxic agents commonly known to inhibit intracellular transport, was investigated by evaluating its effect on the synthesis of Golgi-directed vesicles carrying SM synthase (SMS) and glycosyltranferases (GLTs), the enzymes that reflect restitution of Golgi membrane and determine potential of Golgi to generate sphingomyelin (SM) and an array of the sphingolipids (SphLs) and glycoproteins (GLPs) that govern the cell membrane-specific functions [27]-[31].

Taking into account the findings on the specificity of the vesicular structures rebuilding cell membrane and organellar membranes, we have scrutinized further the impact of ethanol on the aforementioned processes. Its toxicity, as reflected in medically characterized consequences, was tracked from the prime action on the nucleus-initiated processes to its outcomes reflected in formation and delivery of Golgi- and endosome/lysosome- restitution vesicles. Consequently, the principal impact on the terminal apical and basolateral transport was determined by tracing the vesicles containing secretory GLPs and membrane glycosphingolipids (GSphLs) and the vesicles reacting with endosomes. Decline in Golgi-guided transport and the decrease and incomplete glycosylation strongly suggested the attenuation of Golgi restitution and hence impediment of Golgi enzymatic potential to modify vesicular cargo destined to cell membrane [9] [32]-[35]. Alike, the drug-induced inadequate production Golgi restitution vesicles containing SMS implied that inadequate maturation of the basolateral transport vesicles, requiring SM assembly in their membrane, caused inadequate transport to basolateral membrane [30] [31]. Hence, in contrast to the consequences of the depletion of OL SPT [2], the ethanol-induced pathologies were viewed as the results of the nuclear mistiming in the production of the vesicles contributing to the increased potential of the cell catabolic activity which would robustly and viably amplified cellular alterations and apoptotic demise [28]-[31].

As our concept of cell restitution underscores the role of precise tuning of the anabolic and catabolic processes, and the specificity in the vesicles delivery and cell debridement, the evidence presented here provides highly rational and judicious explanation that the large spectrum of ethanol-induced pathologies are initiated by nucleus misguided prompts to refurbish cell organelles and their function. The consequences of balance-derailing drugs in the vitreous experiments allow us to speculate that *in situ* the multitude of cell pathologies are initiated by mistiming of cues determining cell restitution program.

II. Materials and Methods

a) Subcellular Fractionation

For the isolation of cell components the method used herein are well probed techniques described in [2] which allowed us to purify cell and its organelles with minimum breakup of their structure, that cell cytosol was not admixed with fragmented organelles and that cell membranes could be separated from intracellular components.

The cells were prepared from rat liver and gastric mucosa as described previously [2] [22] [26] [32]-[35]. The single cell, incubated in MEM for 3 hours with or without radiolabel, were used for preparation of nuclei [22], subcellular organelles, cell cytosol and cellular membranes [1][2] [36]. In the experiments dedicated to the vesicles synthesis in the presence of drug (ethanol), the translation active control cytosol (CC) or cytosol adjusted to 120 mM ethanol (CEt) were subjected to 30 min incubation with nuclei, centrifuged to separate nuclei and then used in formation of ER-derived vesicles. The preparations of the organelles were rinsed with phosphate buffered saline (PBS), or 0.5M NaCl or urea-PBS, in order to remove the associated residual cytosolic proteins and vesicles that otherwise would remain on their membranes and interfere with analysis of the organelle maternal components. The synthesis of phospholipids (PLs), sphingolipids (SphLs) and protein was assessed using radiolabeled [^{3}H] inositol, [^{3}H] arachidonate, [^{14}C] choline, [^{3}H] serine, [^{3}H] palmitate and [^{32}P] ATP [21] [22] [26] [32] [33] [35]. The cells' ER organelle was incubated with vesicles-depleted CC and/or CEt and specific label named above, and the formed products subjected to separation from organelles as described earlier for organelle-derived biosynthetic transport vesicles [1] [2][9] [13]. The vesicles generated in the control, ethanol-treated and nuclei-conditioned CC and CEt, respectively, were subjected to fusion experiments with Golgi. In turn, thus generated radiolabeled Golgi was subjected to incubation aimed to generate Golgi-derived transport vesicles, followed by separation of the medium containing Golgi-modified transitory transport vesicles destined to endosomes, and/or apical or basolateral membranes. Both incubations that were aimed to generate the ER- and the Golgi-derived vesicles employed vesicles free CC or CEt at concentration of 15 mg protein/ml. The incubation mixture was enriched with components described in [1] [2]. In the experiments dedicated to analysis of the maternal organellar lipid composition and determination of the organelle restitution with newly synthesized radiolabeled ER-derived vesicles, the incubation to generate transport vesicles was repeated in the presence of cytosol derived from CHX-treated cells [1] [37].This step was introduced in order to complete and release from the organelle, the transport vesicles

dedicated for restoration of other than ER, Golgi or endosome organelles. In continuation, the first batch of organelle-derived radiolabeled transport vesicles, whose label was carried from ER transport vesicles reacting with Golgi, was subjected to incubation with endosomes, followed by separation of the endosome and the cytosol containing Golgi vesicles not reacting with endosomes.

b) Preparation of the Control and Ethanol modified Cell Cytosol

The viable cells used for preparation of cell cytosol were homogenized and centrifuged as described previously [1] [2]. After final centrifugation at 100,000xg for 1h, the obtained soluble fraction was adjusted to 15-18 mg protein/ml, admixed with an ATP generating system [2] and referred to as active CC. The vesicle-free cytosol was then admixed with 120 mM ethanol or proportional amount of buffer to which freshly isolated nuclei were added. After 30 min incubation with nuclei, the cytosols were recovered and referred to as control cytosol (CC) and ethanol modified cytosol (CEt).

c) Preparation the Organelles and Membranes derived from CC and CEt Cytosol

The cell membranes and subcellular organelle fractions (mitochondria, ER, Golgi) were recovered from the cold or radiolabeled cells as described earlier [1] [2] [13].The mitochondria and lysosomes were purified from 10,000xg spun fraction [38] [39]-[42]. The lysosomes were recovered from the mitochondrial fraction following treatment that afforded swelling of mitochondria [38] [39] [40] and used for incubation with vesicles derived from CC and CEt. The crude endosomes were isolated from post-mitochondrial supernatant [2] [13] and fractionated on sucrose gradient described in [2]. The pure endosomes were recovered from 35-8% interphase.

d) Assay of the Inner and Outer Serine Palmitoyl Transferase (SPT) of ER

The ER isolated from other cell organelles as described previously [2] washed with 50mM Tris -HCl, 25mM KCl, 5mM magnesium acetate, pH 7.5 (TK buffer) to remove sucrose, suspended in the same buffer with or without 2M urea and incubated on ice for 1h [24]. During this treatment the OL SPT was released from ER and recovered in the 15,000xg supernatant. The IL SPT was retained in the urea-treated ER membranes [2] [24]. Thus obtained fractions of ER containing 50-100 μg protein were used for SPT assays detailed in [2]. After establishing SPT activity in the ER membranes and 2 M urea extracts, further characterization of the fraction containing enzymatic activity was performed to establish whether 120 mM ethanol impacted activity of the urea releasable OL SPT or ER retained IN SPT.

e) Purification of the ER-Derived Vesicles Generated in the Presence of Control or Ethanol-Modified Cytosol

ER- and Golgi-derived transport vesicles were generated in the presence of radiolabeled precursors according to procedure described previously[1] [2][13].The ER or Golgi membranes, mixed with CC or CEt, ATP-generating system, UTP, CTP GTP, fatty acyl CoA and water soluble cold or radiolabeled lipids precursors, were incubated for 30min at 37°C, centrifuged over 0.3M sucrose and treated with stripping buffer at 2°C for 15 min followed by centrifugation at 10,000xg for 10min to separate transport vesicles from ER or Golgi membranes. As indicated earlier, the step of generation of the vesicles from specified organelle was repeated in order to complete and separate synthesized or modified transporters from the maternal organelle. Only the first harvest of transport vesicles was used in the continued experiments. For that, the products separated from maternal membranes were recovered from the supernatant that resulted from centrifugation of the mixture at 150,000xg for 1h. The crude fraction of the transport vesicles was suspended in 55% sucrose, overlaid with 55-30% gradient and centrifuged at 150,000xg for 16 h. The purified transport vesicles were recovered from the gradients as reported earlier [1] [2] [13].

f) Fusion of transport vesicles with ER, Golgi, mitochondria, endosomes and lysosomes

One volume of radiolabeled vesicles (1.3-1.5 mg protein/ml) was suspended in one volume of CC or CHX CC (CEt was used only for the synthesis of ER-derived vesicles) (15mg protein/ml), and added to one volume of cold cell organelles (5mg protein/ml). The reaction was allowed to proceed from 0-30 min at 4°C (control) and at 37°C in the presence of ATP regenerating system [2]. After incubation, the respective organelles were recovered by centrifugation through three volumes of 0.5 M sucrose at 3,000 rpm for 5 min. The ER vesicles recovered from the supernatant after incubation with Golgi were purified on 55-30% sucrose gradient and used in fusion experiments with mitochondria and lysosomes [32][38] [41]. In turn, the Golgi fraction recovered from the first cycle of fusion with ER-derived vesicles generated in the presence of CC or CEt was isolated, introduced to medium generating transport vesicles and, the reaction products of the second cycle of vesicles synthesis, subjected to separation into Golgi maternal organelles and Golgi-derived vesicles. The Golgi-derived vesicles were than incubated with cold endosomes [39]. One volume of the recovered vesicles (0.9-1.1 mg/ml) was suspended in one volume of CC (15 mg/ml) and added to one volume of purified endosomes (5mg/ml). As described for the ER vesicles fusion with Golgi, each reaction was allowed to proceed for up to 30 min and under the same conditions. Finally, the endosomes and the Golgi-

derived vesicles remaining in CC were recovered and their radiolabeled SM and SphL quantitated. At that point, the radiolabeled endosomes and the nonreactive with endosomes Golgi vesicles were subjected to sphingomyelinase (SMase) treatment followed by radiolabeled lipid analysis [1] [2] [43] [44]. In each experiment the fusion of transport vesicles with acceptor organelle was followed by treatment with 2 M urea at 4°C or 0.5M NaCl in order to remove the vesicles which have not undergone *en bloc* fusion. The associated but not fused vesicles were combined with the free vesicles that remained in CC medium. Then, the recovered organelles were centrifuged through 0.5 M sucrose, washed and subjected to radiolabeled lipid analysis. The lipid analyses concentrated on identification of SM, SphL and Cer, the products of Golgi-specific SMS and SM ase degradation, respectively. Therefore, to show SM and Ceramides (Cer), the alkali-susceptible phosphoglycerides (PhGs) were eliminated by subjecting lipid extracts to alkaline methanolysis and then thin layer chromatography along with standards of SM and Cer. In experiments containing radiolabel derived from palmitate labeling, the alkaline degradation products were subjected to one dimensional thin layer chromatography but in two solvent system, first to separate Cer and free fatty acids using mixture of petroleum ethers/ ethyl ethers/acetic acid (80:30:1, v/v/v), while the second solvent system consisting of chloroform/ methanol/ water (65:35:8, v/v/v) was used to identify and quantitate SM that after first chromatography remained at the origin.

g) Apoptosis assay

Quantitative measurement of hepatocytes apoptosis was performed using sandwich enzyme immunoassay (Boehringer Mannheim) directed against cytoplasmic histone- associated DNA fragments [45]. Aliquots of supernatants containing cytosol derived from hepatocytes isolated from controls and alcohol-fed animals [35] or control cytosol and the named subcellular fraction isolated from experiments performed in the presence of CEt was incubated with freshly prepared nuclei, centrifuged to remove the nuclei, and reacted for 90 min at room temperature in the microtiter wells with immobilized antihistone antibodies [45]. After washing, the retained complex was reacted with anti-DNA peroxidase and the immunocomplex-bound peroxidase incubated for 30 min on the plate shaker with 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate reagent for spectrophotometric reading. The values were expressed in apoptotic units per milligram of protein (absorbance at 405nm/mg protein x100). All measurements were conducted on triplicate aliquots derived from each sample.

III. Results

Cell nucleus processes are induced by numerous agents that are capable to impact cell membrane receptors or can penetrate the membrane [10] [46]. Ethanol, one of the later, is readily distributed throughout the body, easily crosses biological membranes and thus instantaneously affects nucleus and virtually all organs and cellular processes [23] [27][29] [32] [47]-[49]. Consequently, its toxicity is linked to the occurrence of many pathological conditions but so far no single mechanism has sufficed to account for the development of a variety of medical problems [18] [23] [47] [48]. Our initial study on ethanol-induced changes in cell function concentrated on ER specific role as the internal base for generation secretory cargo transporting vesicles where the system's synthetic ability was assessed following 30min at 37°C incubation with the drug. These studies demonstrated that the total impact of ethanol was manifested in the 26.4% decline (10,978 vs. 8,080cpm/100 μg protein) in generation of palmitate and serine-labeled ER transporters. Similar trend in ethanol induced cellular performance was determined by quantitation of the secretory glycoproteins assembled in chronic alcohol feeding study which also documented that the posttranslational glycosylation of the secretory cargo decreased drastically [35]. At that stage of our investigations we have not considered the fact that ER-derived vesicles represent precise constructs rehabilitating different cell organelles and that the demand for their synthesis may differ depending on cell derivation, its metabolic status or primary changes that might be induced by the administered drug [1] [2] [9]-[11] [20] [35] [44]. Now, in the context of the investigations that identified spectrum of an organelle-specific ER-initiated transporters [9] [13], the subject confronted here was whether drug such as ethanol affected all, or just the specific group of ER-derived transport vesicles. Particularly, our efforts concentrated on the determination whether drug evoked changes in Golgi-destined products implicated the restitution of Golgi, endosomes, or the cell membrane.

While the widely held view of the acute and the chronic consequences of exposure to ethanol is based on the collective end image of the response to the drug, in this study we have concentrated on ethanol-induced nucleus-initiated processes that impact composition of the cytosol, and henceforth define the consequential events responsible for the production of aforementioned groups of the vesicles. As our concept of cell restitution underscores the significance of nucleus-controlled, balanced responses in the production of the precise quantity and built of the vesicular constructs for every organelle and cell membrane, we put to test an idea that multi componential ethanol toxicity must be initiated in nuclear processes and the aberrant cues released to the

cytosol distort restoration of cell organelles and that culminates in an abnormal cell functions.

To single out and characterize the nucleus initiated cascade of the processes triggered by exposure to ethanol and linked to the synthesis of ER transport vesicles, the cytosol used in assembly of the transporters was admixed with 120 mM ethanol and incubated with freshly isolated nuclei. Thus induced nuclear processes and hence possible alteration of cytosol distinct specificity were measured by the labeling and quantifying the transport products generated in the aforementioned cytosols. Depending on the radiolabeled tracer, (P-choline, or serine), the gradient profiles of the ER-derived vesicles either showed decrease in the total production or were identical with the control (Figure 1). With serine tracer, the labeling of newly synthesized vesicles carrying protein was found to be reduced by 30% (7,224 µg protein harvested from CC-, and 5025µg from CEt-harvested samples), but the serine labeling of the vesicles membrane SPhL core, the Cer, was almost identical. The total label incorporated into the lipids was 19,881 cpm/mg in CC- and 19,757 cpm/mg in CEth-derived vesicles. The lipid label incorporation and the gradient profiles of Golgi-directed transporters suggested that ethanol impacted assembly of the vesicles toan unequal degree, and that was manifested by disproportion in the labeling of the cargo, PhGs and SphLs.

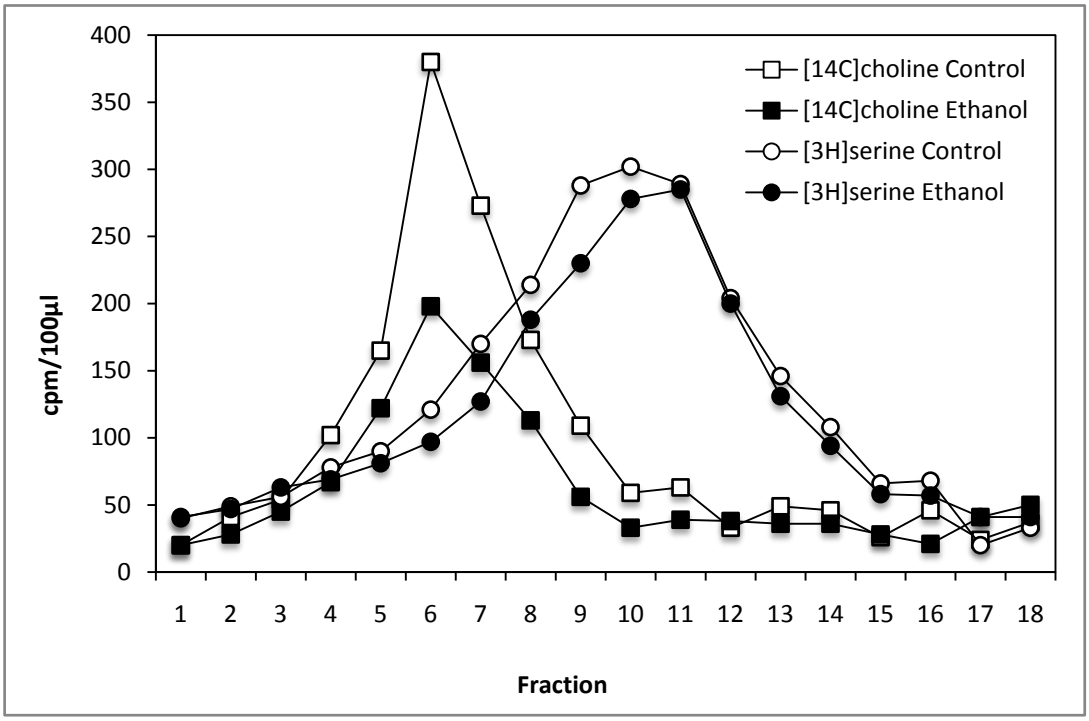
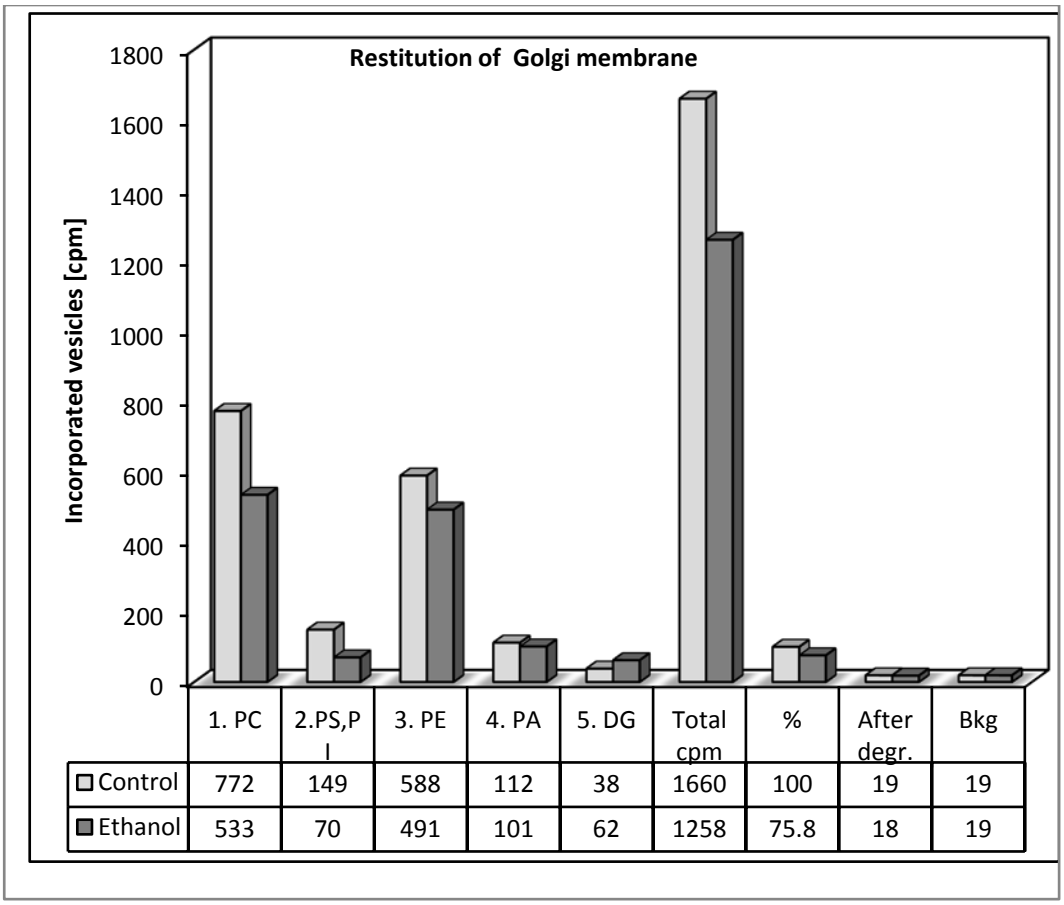


Figure 1 : Quantitative and compositional differences in transport vesicles captured by employing labels identifying P-choline in PLs and serine incorporating into SphLs.

While the entire array of P-choline labeled ER vesicles showed significant reduction in the assembly of the transporters, without P-choline labeled PLs the difference between control- and ethanol-derived ER vesicles was less apparent. Since Cer synthesis was not affected and OL SPT activity was not influenced by its membrane attachment or detachment status [2], we could safely deduce that ethanol treatment was not affecting synthesis of Cer containing ER-derived vesicles destined for endosomes [2]. Rather, with the evidence obtained from earlier investigations of ER-assembled transport vesicles that demonstrated formation of organelle-specific constructs [13], possibility arisen that the spectrum of the ER assembled transporters programmed for apical or basolateral secretion, or restitution of other yet not characterized intracellular organelles, was assembled in modified and the inharmonious quantity. To affirm such likelihood the investigations were carried to determine the relative quantity of the Golgi-, endosome-, and cell membrane-restitution vesicles. The lipids of Golgi membranes labeled by prior incubation with ER-derived vesicles and subjected to consecutive incubation to generate Golgi-derived transport vesicles and then, while depleted of their transitory cargo, evaluated in terms of lipids (derived from ER transport vesicles that permanently incorporated in their membrane as Golgi restitution vesicles)are shown in Figure 2.



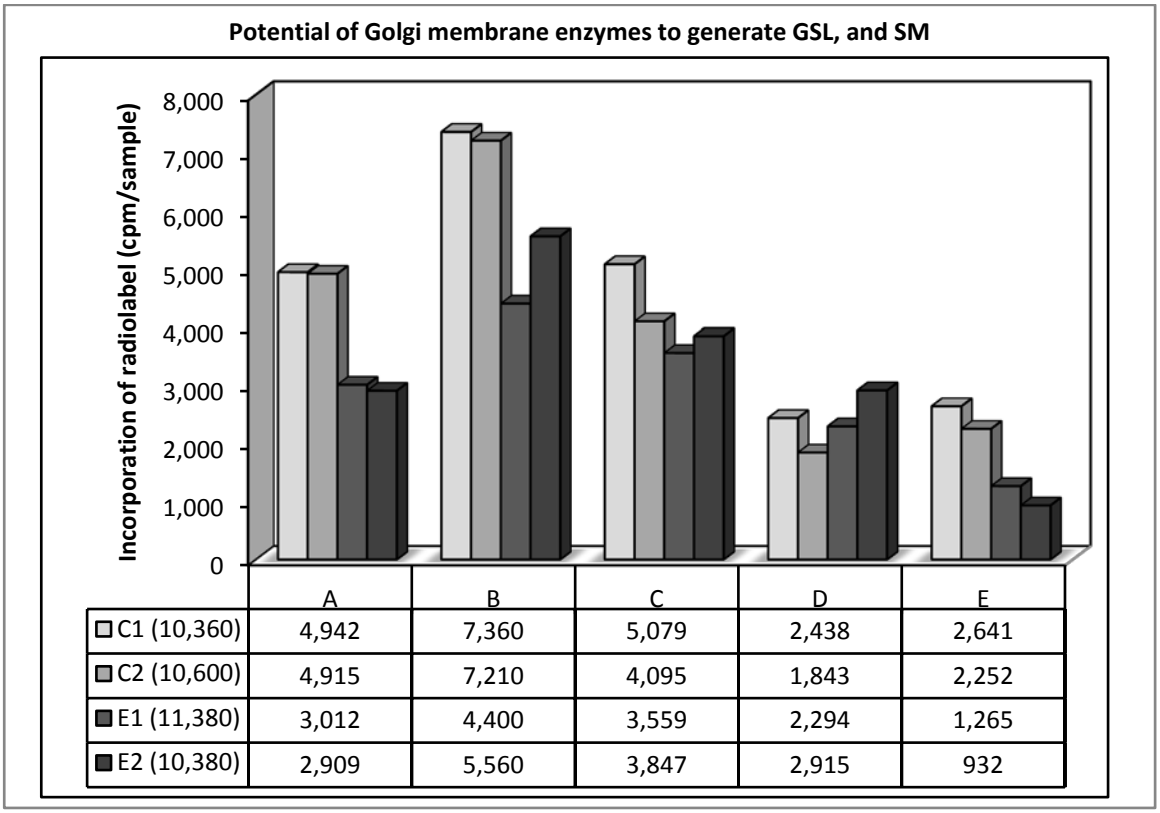
	1. PC	2.PS,PI	3. PE	4. PA	5. DG	Total cpm	%	After degr.	Bkg
Control	772	149	588	112	38	1660	100	19	19
Ethanol	533	70	491	101	62	1258	75.8	18	19

Figure 2 : Quantitative and qualitative differences in restitution of Golgi organelle. The analysis of the labeled lipids that incorporated into Golgi membrane following incubation with ER-derived transport vesicles generated in the presence of unmodified and ethanol-stimulated cytosol. As demonstrated in the analyzed samples, the vesicles incorporating into Golgi membrane constitute of PhGs and glycerides represented by phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidic acid (PA) and diglyceride (DG) without contribution of SphLs.

The analysis of Golgi-derived maternal membranes revealed that Golgi organelle and its restitution vesicles are free of SphLs, and the quantity of PhG-labeled constructs delivered via Golgi restitution vesicles in ethanol derived preparation were reduced by 24.2%. Also, regardless of the sample derivation, (CC or CEt-induced), the Golgi maternal membranes, isolated after repeated incubation and release of the transitory vesicles, were devoid of SphLs and after alkaline degradation of membrane glycerides (**Figure 2**) neither SM, GSL or Cer were found. These results confirmed that reduced radiolabeling of thePhGs in ER-generated transport vesicles reflects diminished production of the vesicles restoring Golgi organelle.

As the potential of Golgi to modify the cargo of the transiting vesicles depends on restitution of the organelle membrane-embedded GLTs and SMS, we have investigated the impact of the reduced rebuilding of Golgi membranes on their activity. By introducing radiolabeled sulfate and comparing its use by the samples of Golgi subjected to incubation with ER transport vesicles carrying equal amount of Cer substrate in CC- and CEt-derived vesicles (**Figure 3, column A**), and by overall production of the SphLs which included synthesis of SM (**column B**), the results showed reduced production of GSLs and SM in the CEth-derived samples. That implied that ethanol-derailed Golgi membranes restitution affected the critical processing of the vesicles destined for cell membrane which was reflected in the drastic reduction in the synthesis of GSphL sulfate, and overall synthesis of SPhLs including SM (**Figure 3, column A, B, respectively**). Thus, based on the lipid compositional analysis of Golgi, and the overall decrease in the vesicles refurbishing Golgi potential to transform ER-derived Cer into GSLs and SM, we concluded that the quantifiable incongruity in ethanol-induced labeling of the Cer-containing ER transport vesicles reflects the fraction representing other than Golgi restitution vesicles. In order to determine which group of Golgi transiting restitution vesicles was affected, our further investigations concentrated on the analysis of the Golgi-

derived Cer-labeled vesicles displaying affinity with endosomes. As illustrated in Figure 3, the amount of Cer-labeled Golgi vesicles was reduced in the ethanol-derived preparations (column C), yet the amount of those with the affinity toward endosomes was higher or equal to that of control (column D). Moreover, the recovery of the vesicles not reacting with endosomes was drastically reduced (column E).



	A	B	C	D	E
C1 (10,360)	4,942	7,360	5,079	2,438	2,641
C2 (10,600)	4,915	7,210	4,095	1,843	2,252
E1 (11,380)	3,012	4,400	3,559	2,294	1,265
E2 (10,380)	2,909	5,560	3,847	2,915	932

Figure 3 : The synthetic activities of the Golgi-specific enzymes utilizing Cer-containing substrate delivered from ER to Golgi through vesicular transport. A-synthesis of GSphL sulphate, B-synthesis of GSphLs and SM, C- the total labeled SM detected in Golgi-generated transport vesicles, D-the labeled SM retained with endosomes following incubation with Golgi-derived vesicles, E- labeled SM in the Golgi-derived transport vesicles not reacting with endosomes. The demonstrated results depict two control- (C1, C2), and two ethanol-(E1, E2) derived preparations, each containing shown amount (in parenthesis) of the labeled Cer.

Thus, compositional analysis of the Golgi-derived Cer-labeled transport vesicles confirmed our initial statement regarding OL SPT activity [2], and showed that the majority of Cer-derived lipids represent endosome-directed transport, whereas the cell membrane directed cargo-carrying transporters are greatly reduced. Using the same amount of Cer labeled Golgi-derived transport vesicles in the reaction with endosomes, we have found that on the average, 34.1 % more of ethanol-derived Golgi vesicles was retained by the endosomes than from the parallel controls (Figure 4).

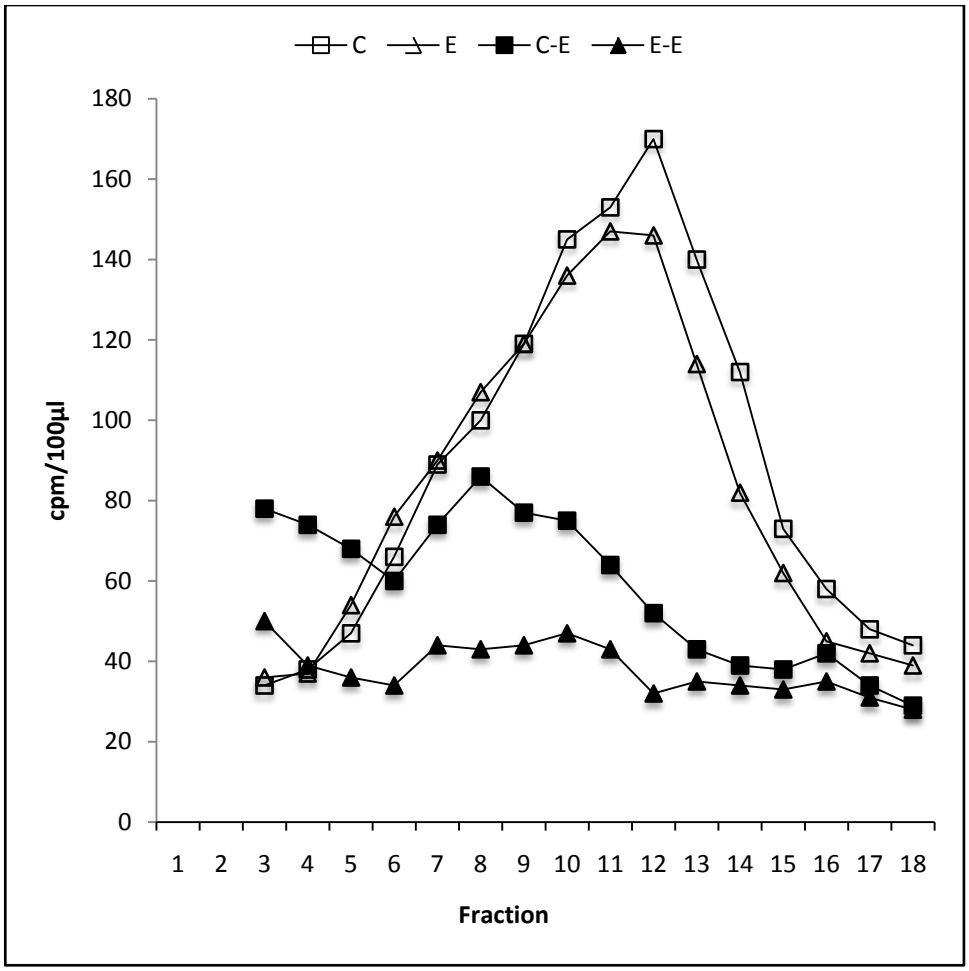


Figure 4 : Golgi-derived transport vesicles reactivity with endosomes. The samples of equally Cer-labeled vesicles from C (C) and CEt (E)before incubation with endosomes (open squares and triangles, respectively) and the fractions remaining free in the cytosol after incubation (closed squares and triangles),were subjected to the sucrose gradient centrifugation. As visualized, the fraction recovered from E samples (EE) was substantially smaller than one from corresponding control (CE). On the average, the CEt-derived preparation (E) consisted of 34% more of the endosome reactive vesicles than the matching control (C).

Thus, this experimental approach gave us strong supportive evidence that the Cer substrate provided in ER-assembled and Golgi-destined vesicles reflected disproportional assembly of the vesicles refurbishing endosome/lysosome organelles andgave us base to speculate that the surplus of Cer-containing vesicles reflects increased production of the endosome directed cargo. Moreover, it confirmed that the Cer is the product of OL SPT, that supplies SM to the outer leaflet of endosome/lysosome membrane. All together, the results suggest that the apparent discrepancy in the ER derived transport vesicles labeled with P-choline showed overall decrease in formation of the vesicles including those responsible for restoration of Golgi. On the other hand, the Cer labeling, as shown in **Figure 1**, demonstrated increased proportion of endosome-directed vesicles which were identified after their release from Golgi and reactivity with endosomes shown in **Figure 4.**

As demonstrated in previous investigations the assembly of the vesicles marked by the SM and GSphLs, is controlled by the provision of Cer on the inner vesicular membrane, and is not affected by the elimination of the ER's OL SPT with which the restitution of endosomes and transport of hydrolytic enzymes have been drastically reduced [2]. In context of these consequences, the results obtained here reveal the drug- induced contrasting outcome; ethanol affects the processes that control translation of the specific mRNA composites aligned with synthesis of SphL controlled by IL SPT. Consequently, the diminished production of the transport vesicles destined to cell membrane debilitates restitution of the cell specific membrane.

While matching amount of Cer incorporated label determined increased quantity of the vesicles with affinity to endosomes (**Figure 4),** further investigations were carried out to determine whether the vesicles carried endosome-specific cargo. The comparison

study between Golgi vesicles delivered SMase contribution to degrade SM substrate by control-derived endosomes with those derived from ethanol-primed samples are depictedin Figure 5. The results depicting the activity of the acidic SMase clearly demonstrate that the ethanol-induced system produced endosome/ lysosomes with higher concentration of hydrolytic enzymes. On the average, the lysosomal SMase potential to degrade SM (EGv minus EC) was 36.4% higher in the CEth-derived samples than those generated by CC.

The outcome of the increased concentration of the catabolic enzymes evoked by the apparent increase in growth of endosome/lysosome organelles was investigated further by performing comparison test of apoptosis induced by ethanol-derived and control preparations. As illustrated in Figure 6, the potential of ethanol-derived samples to invoke apoptosis was 3 fold greater than that of controls.

As demonstrated in Figures 5 and 6, the ethanol-induced Golgi-derived vesicles reactivity with endosomes, the acidic SMase activity, and the degree of apoptosis correlate with the disproportional ethanol-induced restitution of endosomes.

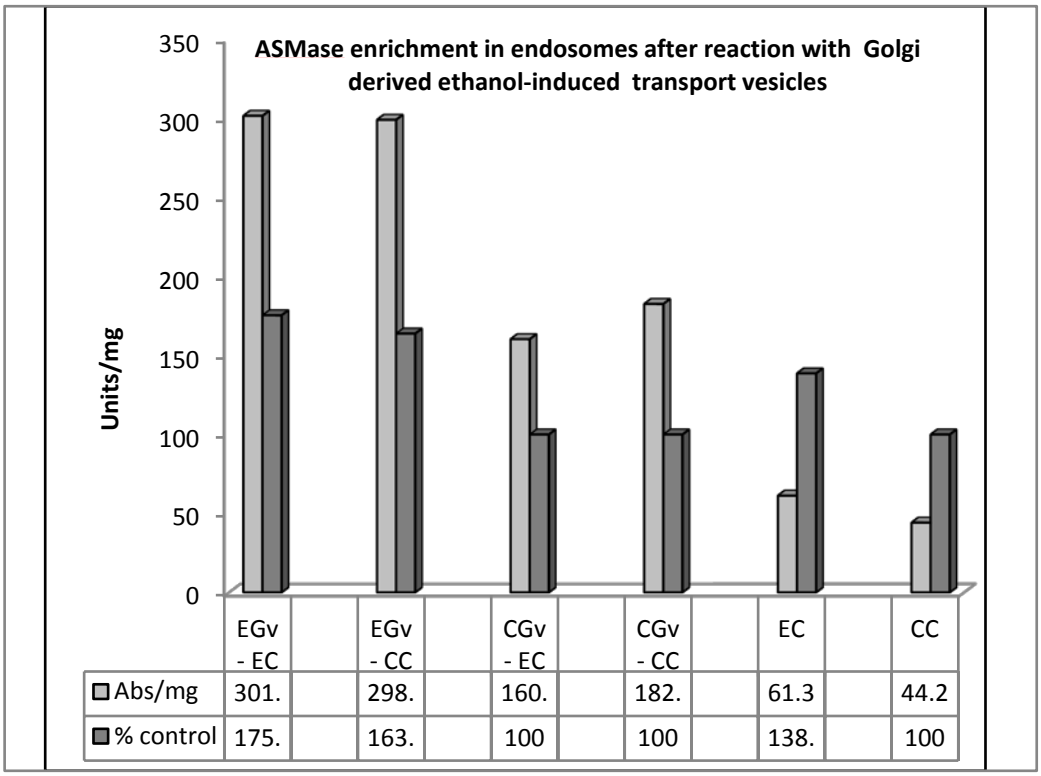


Figure 5 : Comparison of the lipolytic activity of the lysosomal Acidic SMase (ASMase) released from organelles following reaction with Golgi-derived vesicles derived from CEt-and CC preparations. The abbreviations represent Ethanol-Golgi-derived vesicles (EGv), Control-Golgi-derived vesicles (CGv), CEt Cytosol (EC), Control-derived Cytosol (CC).

Assay mixture	Volume used (µl)	ELISA Abs/well	ELISA Abs. average	Abs/ 100 µl	Abs/ 100µg	Induced apoptosis (fold)
EGv + EC	15	0.825 0.876 0.790	0.83	5.540	36.2	8.2
EGv + CC	15	0.899 0.684 0.748	0.78	5.18	34.3	7.8
CGv +EC	15	0.597 0.598 0.615	0.60	4.00	22.1	5.0
CGv + CC	15	0.620 0.620 0.609	0.62	4.1	22.7	5.2
EC	70	0.604 0.828 0.631	0.69	0.99	6.1	1.4
CC	70	0.540 0.739 0.455	0.58	0.83	4.4	1.0

Figure 6 : Induction of apoptosis by the lysosome/endosome preparations following reaction with Ethanol Golgi- and Control Golgi-derived vesicles. As shown, the ethanol derived preparations caused 3fold greater apoptotic demise than the control. The abbreviations describing derivation of the samples are the same as in Figure 5.

The tests whether other cell organelles release to cytosol factors that could contribute to increased apoptotic index were unconvincing. While the ethanol-derived cytosol alone seemed to induce apoptosis to slightly higher degree than control (Figure 6, EC vs. CC), in combination with control-derived vesicles its impact was not observed (Figure 6, CGv+EC vs. CGv+CC). As became evident from the diminished restitution of Golgi membranes and reduced potential of the embedded enzymes to utilize Cer, the query regarding seemingly unaffected synthesis of Cer-containing ER vesicles initially transferred to Golgi has been answered through results of the experiments utilizing endosomes reactivity with Golgi derived vesicles. Together with experiments showing the increased activity of lysosome specific acidic SMase, and induction of apoptosis, the information gathered affirmed that discrepancy between ER-initiated protein-, PhG- and Cer- labeling reflected modified production of Golgi restitution vesicles, decline in the vesicles destined for cell membrane renewal, and substantial upsurge in the replenishment of endosomes and their lytic cargo.

Our experimental approach that relies on specific contribution of lipids in building and restitution of cell organelles and its membrane, not only gave tool to differentiate between vesicles directed to various organelles, but also solidified previous findings that mitochondria and Golgi organelle membranes are free of SphLs [13]. Importantly, and highly relevant to the studies presented here, the approach allowed us to determine the ethanol-induced crucial stages that debilitated Golgi restitution, diminished its potential to transform Cer into SM and adequately glycosylate GSphLs and GLPs cargo [35]. Furthermore, since Cer is exclusively generated in ER membrane during assembly of the Golgi destined transporters, the facts imply that ethanol from the outset of the synthesis divergently influenced the production of the vesicles carrying cell membrane-destined cargo and those assembled for endosomes which in an essence created disequilibrium in restoration of cell and its organelles. Collectively, the results presented above provide convincing argument that the consequences of ethanol-induced nucleus-initiated processes reverberate in the erroneous production of the vesicles restoring Golgi, cell membrane and endosome-specific vesicles. Together, the ethanol-induced derailed repair of the cell may lead to a loss of misguidedly modified cells, or proliferation of the cells with altered glycosylation, appearance, and function.

IV. Discussion

The organization of the key cell identity genes into insulated spaces is recognized as a common trait to all mammalian cell types [5][6] [11] [16] [50] [51]. The continuing challenge is to recognize the remaining critical processes which shape cellular distinct features and depend not only on the principles set in nucleus and ER for the synthesis of protein but on the entire conduit that maintains the cell identity scaffold, the cell

function, and the tissue homeostasis [1] [5] [6] [9] [12] [51]-[53]. Our view, founded on the discovery of cells' transport vesicles specificity [1] [2][9] [13] [32] is consistent with the appropriate regulation of protein synthesis (proteostasis), but advances the concept by extension of the encoded cell-specific protein expression and differences to the next level, one that regulates proteostasis by formation of the nucleus-coined ER-assembled functional segments in form of ER transport vesicles that restore, uphold and maintain cell and tissue homeostasis [1] [2] [9] [13]. The mechanisms that underlie the sustainability of cellular differences are beginning to be elucidated mostly by assessing how these processes change with ageing, cancer, or go wrong in various drug-induced diseases that derail the highly regulated process of such physiological equilibrium (homeostasis) [6] [15] [50] [54]-[59]. But, the global analysis of gene expression, mRNA, or terminal effect of the drug on the specific function have not uncovered their contribution to the multiplicity of yet unmarked steps that may shift the equilibrium in favor of other cellular tasks. As a result, the investigation in numerous laboratories, including our earlier studies on ethanol toxicity, restricted to its effect on cell secretory ability have shown only the cumulative impact demonstrated in the secretory decline of gastrointestinal mucous, hepatic protein, transferrin, and intracellular retention [30] [35] [47]-[50] [55].

Yet the initial and decisive factors influencing processes that reflected alcohol impact on the transport to apical and basolateral restitution and secretion remained unknown. The sight into the intermediary spectrum of the drug-induced toxicity on the secretory activity in the unrelated cells created likelihood that the array of consequences ascribed to alcohol-induced pathologies is initiated at the early and pivotal stage in cell response to the presence of the drug and manifested in specific mRNAs translocation and translation [6] [8] [12] [27] [59] [60]. As ethanol easily crosses all biological membranes and thus affects virtually and simultaneously every organ and biological process, we therefore focused on its primary effects on yet unknown nucleus-instructed processes that dictated generation of the ER-assembled vesicular constructs. Specifically, we have aimed at those manifested in the production of ER-generated transport vesicles that fulfill the structural and functional criteria of the organelles, are responsible for the delivery of apical/basolateral secretion, renovation of the cell membrane, and the restitution of Golgi and endosome/lysosome [1] [2] [6] [14] [17]. Consequently, the ability of ethanol to derange transcriptional and translational regulation initiated in nucleus was explored by exposing isolated nuclei to the cell cytosol with the drug, and then using the nuclei-primed and ethanol-altered cytosol in the synthesis of ER transport vesicles. In our reasoning, the impact of ethanol on nuclear processes should be evident in the release of mRNA assemblies provoked by the drug and be reflected in the make-up of the transport vesicles. In turn, the array of the vesicles assembled in ethanol-induced system could then be verified by determining the Golgi potential to provide transiting transporters with the attributes that direct them to the destination site, and form basic structures that precisely fulfill the cell and organelle designated function [1] [2] [9] [11]-[15] [17].

Specifically, we have aimed at Golgi-specific processing of the ER-delivered vesicles containing SphLs core, the Cer, to produce cell membrane-specific GSphLs and SM and SM-containing vesicles that restore endosome/ lysosome system, the tasks accomplished by Golgi membrane-specific enzymes whose potential rests on the supply of Golgi organelle restoring vesicles [19] [34] [35].As demonstrated herein, the samples calibrated according to quantity of radiolabeled Cer, the marker of Golgi delegated vesicles and utilized by Golgi SMs and GLTs, showed significant differences in enzymatic activity of the Golgi rebuilt with vesicles derived from the cytosol primed with nuclei in the presence of ethanol. Having an equal, quantitavely calibrated Cer-containing fractions, the observed discrepancy in the synthesis of SphLs was not caused by the lack of Cer substrate, but rather by the fact that ethanol-derived vesicles failed to replenish matching quantity of Golgi-specific restitution vesicles delivering organelle explicit SphL-generating enzymes. Indeed, we have demonstrated the impact of ethanol on restoration of Golgi membranes through quantitation of ER vesicles-derived lipids retained in Golgi maternal membranes.

With palmitate label we were able to demonstrate that under the impact of ethanol, the ER released vesicles rebuilding Golgi membranes and hence delivering SMS and GLTs were synthesized in lesser quantity than in controls. The quantitative analysis of the Golgi residual label remaining after prolonged incubation and release of transiting vesicles resulted in the demonstration that Golgi residual membranes recovered from controls afforded samples with larger quantity of radiolabeled glycerides incorporated into its organelle membrane than those from ethanol-derived samples. As demonstrated (Figure 2), the quantity of labeled lipids in ethanol-derived preparations was 24% lower than in parallel controls. Together, the quantitative data on labeled ER vesicles incorporation into Golgi membrane and in P-choline labeled ER-vesicles provided strong support to the belief that ethanol abridged Golgi restitution.

Still, the gap remained in the documentation of Cer-containing vesicles carried to Golgi. In spite of almost equal Cer presence in the ER vesicles carried to Golgi, the lipid composition of the Golgi organelle showed lack of any SphLs in the maternal membrane, the quantity of basolateral transport vesicles containing SM decreased, and the Cer-labeled vesicles were not

retained in Golgi. With plausible explanation of Cer-lacking Golgi-rebuilding vesicles based on the evidence regarding lipid profiles of Golgi residual membranes and Golgi autophagosomes, further studies with Cer-labeled ER vesicles were continued to determine whether Golgi-specific SMSs were equally impeded [2] [23]-[26] [34].

As documented in our earlier studies, the products of Golgi SMSs are specific markers of basolateral transport vesicles containing SM in the inner leaflet of the membrane, and the endosomal/lysosomal transport vesicles with SM in the outer leaflet of the membrane [2] [24].Thus, to distinguish between their activities, the Golgi transport vesicles were subjected to incubation with endosomes, and the amount of the radiolabel retained with endosomes and on unreactive vesicles determined. The results indicated that ethanol-derived Golgi vesicles were up to 34% more reactive with endosomes than corresponding controls. Moreover, the SMase activity of the ethanol-derived endosome were on average 50% higher than in controls. Hence, solid support to the argument was gained that while the restitution of Golgi was impeded, the endosome-directed transporters were not affected and their SMase delivered in substantially higher quantity than that for controls.

Thus, the harmful effect of ethanol outspread beyond inconsistent maintenance of Golgi, beyond Golgi capacity to modify transiting vesicles and their secretory cargo dedicated for cell membrane [1] [2] [25][26][35] but into yet unknown site reflected in the excessive building of the degradative potential of endosomes/lysosomes [47] [49]-[52]. Is it passible that such imbalance in generation of degradative potential of the cell and failure to maintain Golgi and cell membrane generate condition to evoke apoptosis [33]-[35] [45]? While we do not have a direct individual factor that would explain that ethanol-induced delivery of the endosome-reactive vesicles stimulates apoptosis, it is certain that disproportional, not homeostatic release of vesicles that restore components of the cell is creating different attributes in the cell than those created in control, and provide potential for the endosomes to increase catabolic lysosomal processing. Also, based on the presented evidence, we could conclude that ethanol action on nucleus alone, as determined by the activity of nuclei-exposed cytosol in the assembly of ER-transport vesicles, impairs controlled contribution of the restitution vesicles rebuilding Golgi and that reverberates in cell membrane's restitution, cell's secretory activity, and the facilitated lytic potential of the endosomes that may culminate in harming cell vital processes.

Our study, could not support conclusions reached by other investigators that ethanol induces protein retention in Golgi [59], yet the fraction destined for basolateral secretion was substantially smaller than the control suggesting that basolateral transport, just as apical transport was diminished. Is it possible that cytosol houses albumin- and Cer- containing undeliverable vesicles? At this stage of the investigation we cannot disprove or accept the findings since our investigation of the possibility that Cer contribute to apoptotic demise of the cell or that such vesicles exist, have not provided evidence to such an end. All the while the documented events point to the ethanol-induced nuclear release of markers to cytosol which induce processes producing disparity in cell restitution.

The findings on the disparity in cell membrane restitution and delivery of the functional attributes to the membrane, however, are supported by an ample of evidence. The earlier studies demonstrated ethanol-induced functional derailments by inadequate glycosylation of GSphLs, GLPs and reduced transport of albumin [31] [34] [35].The arguments on glycome modification are solidly supported by the studies of chronic alcoholism consequences in the development of mucin glycans, multitude of the changes in glycosylated species of blood-contained GLPs and pathological aftereffects reflected in fetal alcohol syndromes [18]-[20] [23] [28] [30] [34] [35] [59]-[61]. Obtained results inadvertently are linked to the diminished potential of Golgi to modify the transiting transporters and their cargo. The further effects of the derailed delivery of the secretory products, potentiated by the modified appearance of the cell membrane containing inadequately glycosylated GSphLs and GLPs, consequently contribute to the major ethanol-induced aberrant activity of the cells that rely on the specific interaction of their glycome arrays. In each case, the feature that dominates changed appearance of the secreted GLPs, or cell membrane GLPs, or cell membrane GSphLs is the decline in glycosylation. The malfunction is not specific for the failure of one, the pivotal GLT, but all those specific for cis- to trans- Golgi membrane residing enzymes [61][62] [63]. It may be argued that some glycosylation stages are affected to higher degree than other, but it should be remembered that sequential processes of glycosylation will be propagated on the account of deficits in generation of suitable substrate, lack of specific glycosidic determinants or specific ganglioside core structure. It is evident that the manifested changes in glycosylation, determined in an *in vitro*, or chronic studies, are not resulting from the lack of suitable monosaccharide substrates which are provided in the same amount in alcohol and control samples, but clearly reflect the diminished potential of Golgi to glycosylate vesicular membrane and the secretory cargo.

Although some studies suggest that basolateraly secreted cargo, including albumin, remains in Golgi [59], the data on the lack of Cer in Golgi argue against retention of Cer-containing basolateral transporters. And, it is abundantly clear that the albumin carrying basolateral transporters are not rejected prior to

fusion with Golgi, since their glycosylated membrane and glycosylated cargo that reflect intra-Golgi modification are partially accomplished [35] [60]. Thus, it is possible that SM-lacking basolateral transporters remain in the cell cytosol and with prolonged alcohol presence their build-up may contribute to the enlargement of the hepatocytes [31] [47] [49] [59] [62] [64]. Certainly, the feature that determines fusion of the transporters with basolateral membrane is somehow connected with presence of SM in their membrane, without which the vesicles miss the mark. process fails. This attribute of basolateral delivery, sets the process apart from one completing apical delivery, which is accomplished in spite of partial glycosylation of the membrane intercalated GSphLs and GLPs. Therefore, it is possible that deficiently restored basolateral membranes, or inadequately glycosylated cell membranes respond differently to their endocytotic turnover, while the deficits in restoration of Golgi may cause ultimate disappearance of the organelle.

While we cannot provide conclusive evidence explaining all the above described effects, our findings allow us to infer that each of these ethanol-induced outcomes, is initiated by disruption of an equilibrated, cell specific release of the vesicles that prevents organellar disrepair, regulates cell membrane activity and thus maintains fidelity of cellular homeostasis. By extricating nuclear processes, reflected in the synthesis of cellular transporters, we were able to gain insights into relationship between transcriptional control of cell identity by the factors released to the cytosol that drive the expression of key cell identity genes reflected in the transport, timely repair of the organelles, and retention of the equilibrium between restitutive and degradative processes. While the onset of ethanol toxicity depicted in this experimental paradigm may initiate changes at three unseparable stages (transport from cytosol to nucleus, intranuclear processes, and transport out of nucleus) it is certain that the nucleocytoplasmic transport modifications lead to disproportion in the synthesis of organelles-specific vesicles.

Taken together, it becomes evident that the initial nuclear response to the presence of alcohol reflected in the synthesis of multiplicity of transport vesicles can be manifested in every part of the cell structure and function, which in turn allows to explain majority, if not the totality, of the ethanol-induced pathologies of various organs. While it is factual that cytosol derived from ethanol-treated hepatocytes increases cell apoptotic activity, that ethanol toxicity in nucleus evokes disproportions in the assembly of ER to Golgi transporters reflected in an excessive growth of endosome/lysosome, the final conclusion that inadequate cell membrane turnover is responsible for increased apoptotic activity still await further investigation. Particularly, in order to make well grounded conclusion, we must also evaluate the ethanol-induced changes in the cytosol as reflected in the mitochondrial vesicles assembly and their contribution to overall outcome of ethanol toxicity [13].

Collectively, the investigative approach utilized in the presented studies allowed us to decipher the following unknowns: (1) Golgi organelle is free of SphLs which suggests that intracellular organelles differ not only in their protein but also in lipid composition. This has been already established in the case of mitochondrial membranes composition [13], (2) the initial impact of ethanol toxicity is translated into decreased restoration of Golgi, manifested in the lessened potential of its enzymes responsible for assembly of cell membrane SphLs and GSLs, and reduced intercalation of the phosphoglycerides-containing Golgi-specific restitution vesicles, (3) increased synthesis of endosome-directed transport vesicles and an excessive growth and enzymatic potential of endosomes/lysosomes, and (4) reduced output of cellular cargo and diminished repair of cell membrane. Thus, in the final outcome, ethanol caused inadequate posttranslational modification of the cargo relying on Golgi-specific processing, catabolic disproportion reflected in hydrolytic activity of ethanol exposed cells, and the cells with inadequately glycosylated GSL and GLPs that impair and modify their functional characteristics. The above described findings imply that in nucleus, ethanol transmits information that is reflected in misreading of restitution cues, thus causing drug-induced disparity in the cell restitution and debridement.

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